The Role of EXOSOMES

in reperfusion injury and cardio-protection

MD (Res) Thesis

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Declaration

I, Debashish Das, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The mechanism by which remote ischaemic preconditioning (RIPC) exerts its cardio-protective effects is not fully understood. Exosomes are lipid bound membranous vesicles that range from 30-100nm in size. With increasing interest in the scientific community about these vesicles as a mode of intercellular communication, in this project we explore the possible mechanistic role of human plasma exosomes in RIPC. We first developed a consistent and reliable model for the isolation of human plasma exosomes through a process of differential centrifugation and ultracentrifugation. Further scientific models for biomarker analysis and quantification of the isolated human plasma exosomes were developed and validated through western blotting and flow cytometry and nanosight analysis. In addition we successfully developed a protocol for the isolation of microRNA from our human plasma exosomes. Confident with our isolation and biomarker analysis models we investigated the content differences in human plasma exosomes isolated after a RIPC (3 cycles of fore arm cuff inflation and deflation) and compared those to control human plasma exosomes. Our results show that RIPC lead to a rise in plasma exosome concentration with a peak rise in exosome concentrations occurring only after the last 5 minutes of cuff deflation (reperfusion) in the 3 cycles of the RIPC protocol. We probed both RIPC and control human plasma exosomes for known pro-survival protein kinases of the RISK pathway (P13K, MEK 1/2, AKT, ERK1/2) though were not able to detect these in any sample. We then tested the hypothesis that the cardio-protection conferred through RIPC is mediated through circulating plasma exosomes. RIPC and control human plasma exosomes were added to an invitro model of ischaemia reperfusion using rat cardiomyocyte cells. Interestingly our results demonstrate that human plasma exosomes are cardio-protective against ischaemia reperfusion injury irrespective if they are control or RIPC plasma exosomes. The cardio-protection conferred by human plasma exosomes is comparable to Insulin, a known cardio-protective agent, used as our positive control. These results suggest that plasma exosomes in general are cardio-protective and may not have a mechanistic role in the cardio-protection conferred through RIPC. Further experiments must be carried out to ascertain the exact mechanism through which human plasma exosomes confer cardio-protection.

Acknowledgments

I would like to express my deepest thanks to my supervisors Professor Derek Yellon and Dr Sean Davidson. You have both supported me without question through out this process and have provided inspiration and guidance in times of need. I would like to thank you for your continuous encouragement as well as your patience and for allowing me to grow as a research scientist. Your advice on both research as well as on my career have been priceless. A special thank you to Dr Jose Miguel Vicencio and Dr Anastasia Kalea for your collaboration, guidance and mentoring. Finally I would also like to thank all my colleagues and fellow researchers at the Hatter Cardiovascular Institute for all the stimulating conversations as well as banter at lunch times, but most importantly making my time in research fun.

Table of Contents

List of Figures

List of Tables

CHAPTER 1

Introduction

Epidemiology and Burden of Cardiovascular Disease

Cardiovascular disease (CVD), despite the advances of modern medicine and reduction in mortality rates, remains the number one cause of death globally. Figures from The World Health Organization show an estimated 17.1 million deaths from CVD in 2004 alone with a predicted rise to 23.6 million by 2030 [1]. This pattern is mirrored in UK mortality figures, with 180,000 deaths in England and Wales (2010) attributed to CVD [2]. Around 46% of these deaths were due to coronary heart disease (CHD) and 28% caused by a stroke [2]. CHD is still the leading cause of death in the UK resulting in one in five male deaths and one in 6 female deaths. A study in 2002 estimated the total annual cost of all CHD related burdens as £7.06 billion, more than any other diseases for which comparable analyses have been done [3]. The most recent figures published by the British Heart Foundation estimate the cost to the NHS of CVD related diseases to stand at around £8.6 billion in 2009 with an overall cost to the UK economy of £19 billion a year taking into account productivity losses and cost of informal care [4].

Coronary Heart Disease (CHD)

CHD is the most common cause of heart disease worldwide. In the early 19th century, acute myocardial infarction (AMI) was generally regarded as a medical curiosity. In 1880 it was Carl Weigert who first suggested the association between coronary occlusion and AMI. CHD arises from the accumulation of atherosclerotic plaque causing vascular dysfunction and resulting in compromised myocardial perfusion. CHD presents itself through a clinical spectrum of angina through to the acute occlusion of a myocardial infarction. The reduction in lumen size due to atheroma leads to a perfusion

demand and supply mismatch that is exacerbated by exercise leading to angina. AMI occurs when there is an acute plaque rupture with subsequent thrombus formation resulting in total occlusion of the coronary artery. Up until 1950, the treatment for AMI was largely focused on palliation rather than curing the underlying process. In the 1970's Marako and colleagues first identified, in animal studies on dogs, that reperfusion of the occluded coronary artery would reduce the extent of myocardial damage[5 6]. In 1976, a Soviet team led by Chazov showed intra-coronary administration of streptokinase in patients with AMI within 4 hours of coronary occlusion, restored patency within approximately 50 min. This effect was not seen, however, when the duration of occlusion exceeded 10 h. This work has set the basis for all future developments of early myocardial reperfusion therapy for the treatment for AMI [7].

Ischaemia Reperfusion Injury

Primary percutaneous coronary intervention (PCI) and thrombolysis are currently the gold standard treatments for patients with an AMI. Paradoxically, however, the return of blood flow can also result in additional cardiac damage referred to as reperfusion injury (RI) and thus reducing the benefit of any reperfusion treatment undergone. Jennings and colleagues, first described RI, in 1960 through experiments with canine hearts which were subjected to coronary occlusion and reperfusion [8]. They showed that reperfusion appeared to accelerate the development of necrosis. Reperfusion injury manifests clinically as four types of cardiac dysfunction; myocardial stunning, which refers to the reversible reduction in contractility of the heart following reperfusion that is not accounted for by tissue damage or reduced blood flow; reperfusion arrhythmias, which are common in patients who have had their coronaries re-opened either by thrombolytic therapy or primary percutaneous intervention (PCI) and is thought to be mediated by mitochondrial dysfunction [9]; the no-reflow phenomenon, where despite opening of the previously occluded coronary artery blood fails to reperfuse the ischaemic area. This is thought to occur as a result of micro vascular damage [10]. The last manifestation of ischaemia-reperfusion (IR) injury is termed lethal reperfusion injury. This is described as myocyte cell death due to reperfusion itself [11].

Pathophysiology of ischaemia and reperfusion injury

Despite reperfusion being necessary to salvage ischaemic myocardium, reperfusion initiates a cascade of events that lead to further death of previously viable cells. Initially ischaemia results in cessation of oxidative phosphorylation and inhibition of mitochondrial ATP synthesis. As a result the ischaemic cardiomyocyte must rely on

anaerobic glycolysis for its ATP supply. Anaerobic glycolysis leads to a buildup of lactate resulting in the acidification of the cytosol. The cell extrudes the H^+ ions in exchange for Na^+ via the sarcolemmal Na^+/H^+exchanger causing a rise in intracellular sodium. The high sodium concentrations cause the Na^+-Ca^{2+} exchanger to work in reverse, which drives a slow increase in cytosolic and sarcoplasmic reticular Ca^{2+} concentrations. Reperfusion causes an acceleration of this process causing a further rise in sarcoplasmic Ca^{2+} which adds to the already Ca^{2+} overloaded cell. Ischaemia also causes a reduction in cytosolic pH that inhibits myofibrillar contraction, however reperfusion rapidly restores normal extracellular and intracellular pH and thus removing this acidic inhibition to contraction. The normalization of pH together with the rise in sarcoplasmic Ca^{2+} results in myocyte hypercontracture [12–15].

Mitochondria play an integral role in the pathophysiology of ischaemia- reperfusion injury. The calcium overload from ischaemia and reperfusion causes the ordinarily impermeable mitochondrial membrane to allow the free movement of protons across the membrane [16]. This change in permeability is largely thought to be mediated via a non-specific pore in the inner mitochondrial membrane called the mitochondrial permeability transition pore (MPTP)[17]. The opening of the MPTP results in mitochondrial swelling, outer membrane rupture and irreversible mitochondrial damage leading to necrosis [18]. The production of detrimental reactive oxidative species (ROS) is a well-established consequence of IR which may be a consequence of mitochondrial damage [19,20]. Free radicals damage myocytes directly by altering membrane proteins and phospholipids. Additionally they trigger cellular injury through stimulating apoptosis and further stimulating opening of the MPTP, which then releases further free radicals in a catastrophic feedback [21,22].

Other factors such as aggregation of leukocytes and inflammatory mediators, platelet and compliment activation are also thought to play a role in the pathophysiology of ischaemia - reperfusion injury, although this is more relevant at later stages after the initial injury [21,23]. The release of leuckocytes into the extravascular space releases further elastases and proteases that destroy the cell membrane and cause cell death[24]. It is also thought that along with compliment activation neutrophil accumulation may contribute to the “no-reflow” phenomenon[25,26].

Damage to cardiomyocytes from IR injury is principally through necrosis, however apoptosis and the activation of pro-apoptotic signalling pathways are thought to contribute to reperfusion injury at later stages[27]. Studies carried out on rabbits have shown markers of apoptosis in reperfused tissue but not in either normal or ischaemic

tissue [27]. Additionally further animal studies have shown that the addition of an apoptosis repressor reduces the extent of IR injury[28].

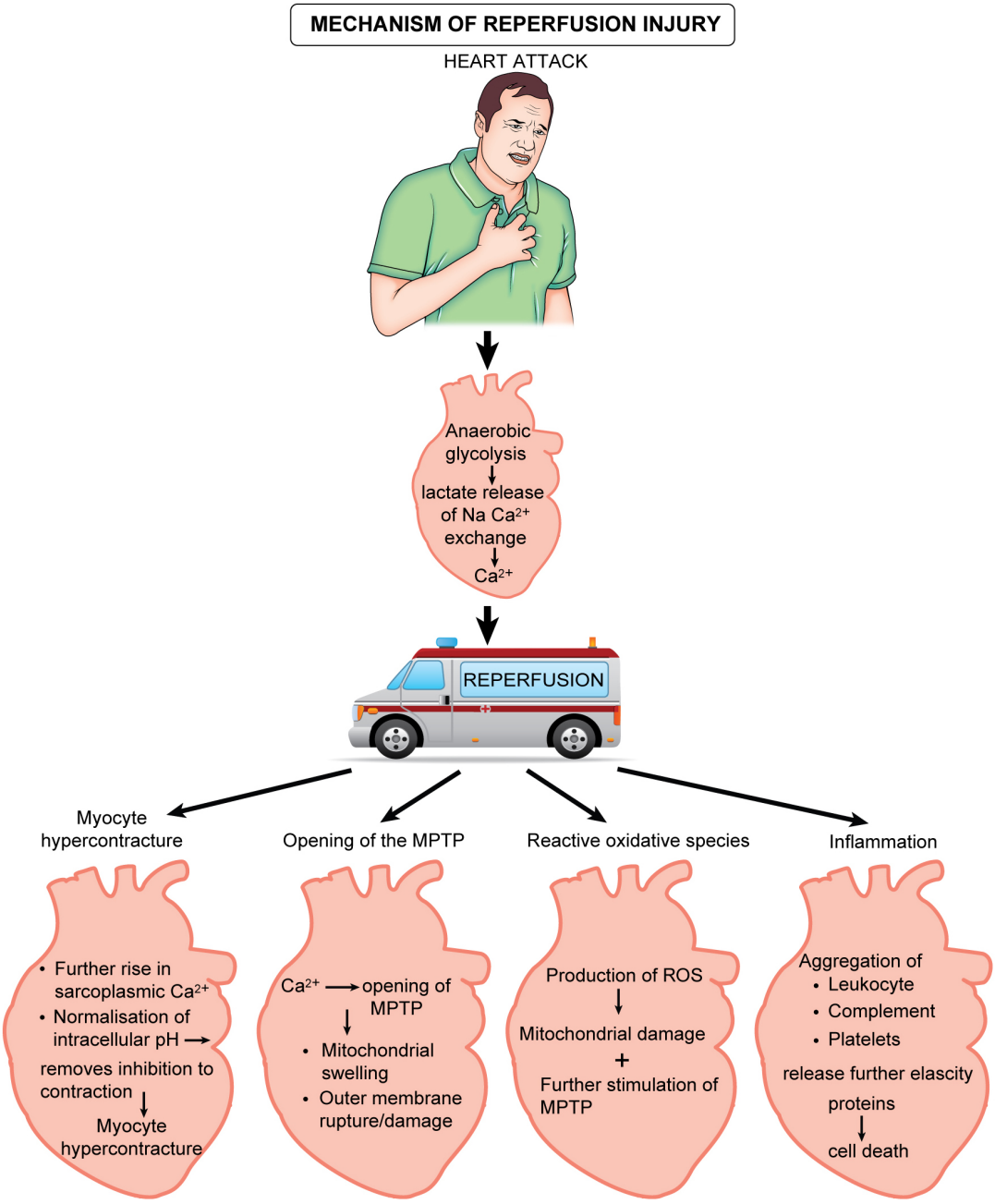


Figure 1 | **Mechanism of IR injury**: IR leads to calcium influx and opening of the MPTP resulting in myocyte hypercontracture, opening of the MPTP and myocyte necrosis through the release of ROS and inflammatory mediators.

Ischaemic Preconditioning

Ischaemic preconditioning is an intrinsic mechanism that protects a target organ from injury and cell death that would result from ischaemia and reperfusion. Murry et al first described this process in their seminal paper in 1986 [29]. The study involved anaesthetised dogs subjected to four 5-minute periods of coronary artery occlusion followed by reperfusion. The dogs were then subjected to a more sustained 40-minute period of coronary artery occlusion. When compared to controls i.e. dogs that did not receive the preconditioning stimulus, the preconditioned dogs had significantly reduced infarct sizes. Prolonged ischaemia leads to depletion of ATP and myocyte necrosis. Murry et al showed that ATP levels fell only during the first cycle of IR and during subsequent ischaemic episodes ATP levels were preserved [29]. Since the publication of this paper ischaemic preconditioning has been shown to be effective in reducing infarct size across a multitude of animal species. These species now include dogs [29], pigs [30], sheep [31], rabbits [32], rats [33], marmots [34] and ferrets [35].

Mechanisms of Ischaemic Preconditioning

Ischaemic preconditioning can be sub divided into two phases early and late. Early or Classical preconditioning occurs immediately after the preconditioning stimulus and its protective effects last for 1-2hrs [36]. Late or delayed preconditioning refers to a “second window” of protection (SWOP), where the cardio-protective effects of preconditioning disappear after 2 hours yet reappear 24 hours later [37,38]. It was Downey and colleagues who first described in rabbit hearts the triggering of protective mechanisms of ischaemic preconditioning via the adenosine A1 receptor [39]. Since then it is accepted that protection is a receptor-mediated process. In the early phase of ischaemic preconditioning adenosine, bradykinin and opioids are released [40]. These substrates bind to their G-protein receptors on the myocyte sarcolemmal membrane. The occupation of these receptors leads to activation of pro-survival kinases that confer cardio-protection. These pro-survival kinases, phosphatidylinositol-3-OH kinase (PI3K)-Akt and the p42/p44 extracellular signal-regulated kinases (Erk1/2) have been termed the reperfusion injury salvage kinase (RISK) pathway and play an important target for cardio-protection [41]. The RISK pathways result in the opening of the ATP-dependent mitochondrial potassium channel and conversely prevent opening of the mitochondrial transition pore and thus convey cardio-protection [42]. The mechanism of cardio-protection through classical preconditioning is complex. In addition to the RISK pathway additional pathways have also been suggested to play a mechanistic role in cardio-protection such as the GPCR/NPR-AKT-eNOS-PKG pathway [43] and the gp130-

JAK-STAT pathway [44]. The mechanism of delayed preconditioning shares many similarities with classical preconditioning. However delayed preconditioning appears to be dependent on the triggering of ROS [45] and nitric oxide which go on to facilitate de novo protein synthesis of distal mediators such as iNOS and COX-2. These mediators are thought to mediate the cardio-protection seen 24 hours after the initial preconditioning stimulus. Heat shock proteins such as HSP27 [46], HSP70, the transcription of eNOS have been shown to play a cardio-protective role in delayed preconditioning [47]. Work done by the Yellon's group has shown the activation of the RISK pathway at the time of reperfusion protects the heart from IR injury [48]. This presents an opportunity for clinical translation of cardio-protection. Work done by Zhi-Qing Ahao et al, using anaesthetized dogs which had their LAD ligated and reperfused, showed that dogs that had intermittent cycles of reperfusion and occlusion prior to full reperfusion (Post-conditioning) were similarly cardio-protected when compared to dogs that had the classical preconditioning stimulus prior to infarction [49]. Ischaemic post conditioning has shown to share common signalling pathways as ischaemic preconditioning. The finding of cardio-protection from ischaemic post-conditioning therefore suggests there still a window of protection that can be utilized even once infarction has taken place.

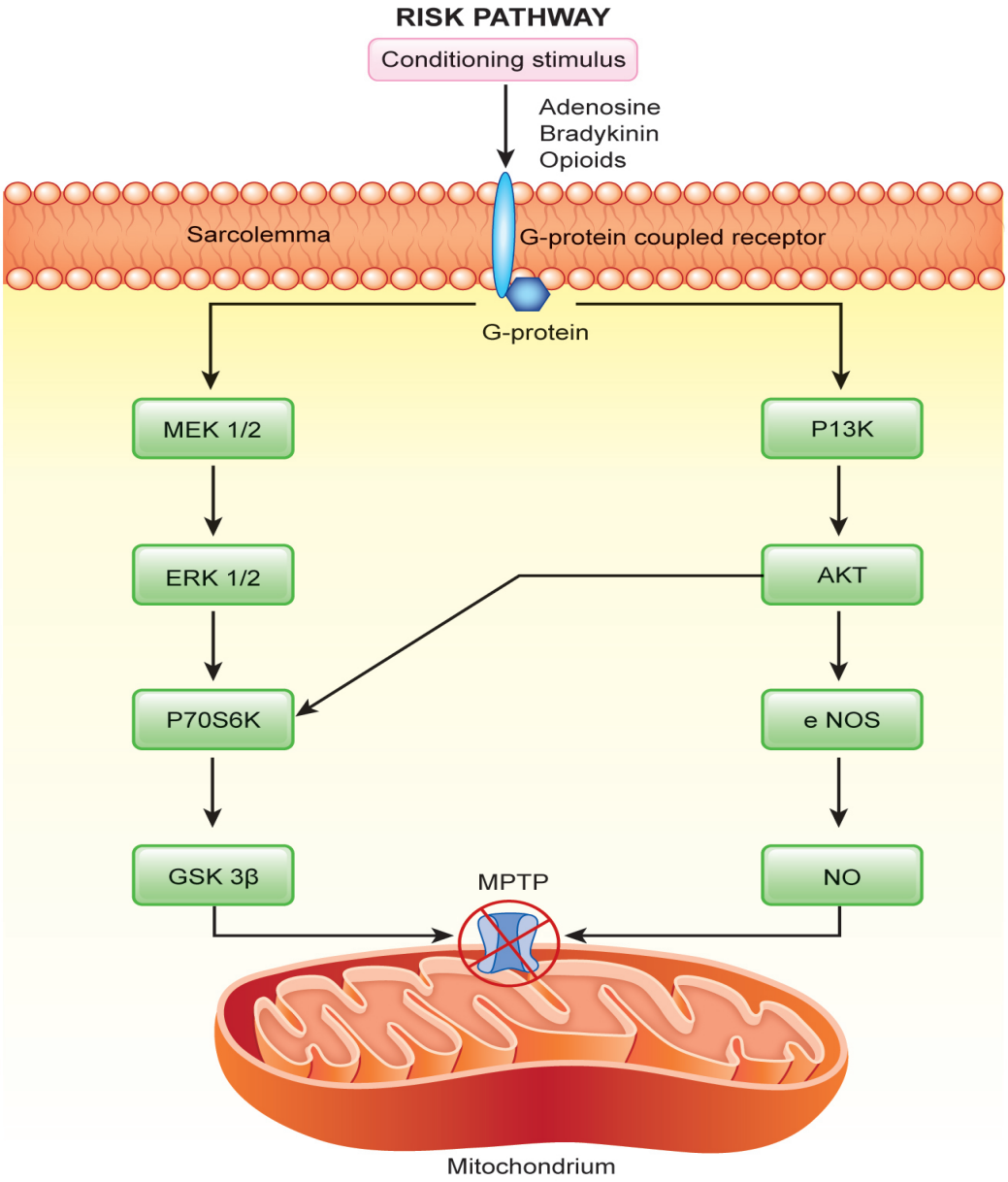


Figure 2 Diagram of RISK pathway and its effect on the mitochondrial permeability transition (MPT) pore. Conditioning stimulus exerts a cardio-protective effect through the activation of G protein-coupled receptors. This leads to activation of PI3K-Akt) and MEK1/2. Further downstream these further activate ERK ½, AKT, ENOS P70S6, and GSKβ. This cascade of pro-survival kinases leads to the inhibition of MPTP conferring cardio-protection.

Remote Ischaemic Preconditioning

A group led by Przyklenk examined the hypothesis that brief episodes of ischemia applied in one coronary vascular bed would confer cardio-protection from infarction in another coronary territory. In anaesthetized dogs, the group were able to show that brief episode of ischaemia and reperfusion in the LAD territory would reduce the infarct size from a subsequent circumflex coronary artery infarction [50]. This for the first time opened up the possibility of protecting the heart through conditioning another distant organ or non-cardiac tissue. Subsequent studies have shown the ability to protect the heart from IR injury by applying the preconditioning stimulus to other organs such as the kidney or small intestine. This phenomenon is now termed 'remote ischaemic preconditioning' (RIPC) [51 52]. While these findings of are extremely exciting their clinical translation has been limited by the invasiveness of intervention needed. The critical break through resulted from the discovery that brief periods of transient limb ischaemia could also exert similar cardio-protective effects of RIPC. The first discovery was made by Birnbaum and colleagues, where they showed that restriction of blood flow to lower limb skeletal muscle and pacing of the gastrocnemius muscle prior to myocardial infarction in rabbits reduced infarct size 65% [53]. Oxman and colleagues further developed this concept and went on to show that by simply applying a tourniquet to the hind limb, inducing 10 min of limb ischaemia in an IR model, significantly reduced reperfusion arrhythmias in rat hearts [54]. However the discovery that limb ischaemia delivered easily and non-invasively by means of inflation of a blood pressure cuff exerting similar cardio-protective effects of RIPC has paved the way for its translation into clinical practise. MacAllister and colleagues undertaking experiments on human volunteers pioneered this area of research. In their studies volunteers were subjected to brief limb ischaemia, achieved through the inflation of a blood pressure cuff to 200mmHg. Volunteers were given 3 cycles of 5 minutes of limb ischaemia (cuff inflation) followed by 5 minutes of reperfusion (cuff deflation) [55]. The contralateral arm was then subjected to a 20-minute ischaemic period through blood pressure cuff inflation and the subsequent endothelial dysfunction analysed. Their results showed a reduction in endothelial dysfunction in those who had undergone a RIPC stimulus on the other arm [55] . Similarly with classical preconditioning, RIPC in its clinical translation is restricted to elective cardiac surgery where the index ischaemia can be predicted. Like ischaemic post conditioning, RpostC, where transient distal ischaemia is applied after infarction but prior to reperfusion, also offers the possibility of applying this cardio-protective strategy to patients presenting with an AMI. Encouragingly using the same model MacAllister and colleagues were also able to show similar results with RPostC as well as a second window of protection 24-48hrs later similar to preconditioning

ref. Andreka and colleagues explored the use of RPostC in pig hearts further. RPostC was induced in pigs by four 5 minute cycles of blood pressure cuff inflation applied to the lower limb immediately after inducing a left anterior descending artery MI. Results showed a 26% reduction in infarct size when compared to their control group [56].

Myocardial mechanism of RIPC

The mechanism by which RIPC exerts its cardio-protective effects is not fully understood. Many of the signaling pathways implicated in pre and post conditioning have also been suggested to play a role in RIPC [57] [58]. However it is still uncertain whether RIPC results in activation of pro-survival kinases of the (RISK) pathway and the subsequent inhibition of the mitochondrial permeability transition pore (mPTP), as in IPC and IPOST [59]. Heidbreder and colleagues tested the hypothesis that mitogen-activated protein kinases (MAPKs) JNK, p38 and Erk1/2 play a mechanistic role behind the cardio-protection offered from limb-mediated RIPC in rats. Their results demonstrated a loss of cardio-protection in RIPC treated rats after the addition of MAPK inhibitors SB203580, PD98059, and SP600125. Additionally RIPC resulted in a considerable increase in phosphorylation of ERK1/2 and JNK1/2 proteins in the small intestine whereas it did not alter the MAPK phosphorylation state in the myocardium [60].

RIPC stimulus of a distal organ is thought to result in a release of humoral factors that are transported to the heart which in turn result in the activation of transduction mechanisms within the cardiomyocyte. These humoral factors bind to plasma G-protein coupled receptors, which activate intracellular kinases such as ERK1/2, PI3K/Akt, PKC-ε, etc. In particular studies have shown myocardial PKC-ε activation in response to binding of the bradykinin B2 receptor and in response to the mediator CGRP [61] [62]. Wolfrum and colleagues investigated the mechanism further and were able to demonstrate the loss of cardio-protection from RIPC by the non-specific PKC blocker, chelerythrine [63] [64] [65]. Humoral factor binding to G-protein coupled receptors also leads to activation of other signaling components such as nitric oxide (NO) and the mitochondrial K_{ATP} channel, although it must be recognized that the very existence of this channel remains controversial [66] [65] [67] [68]. There have been several studies that have linked the opening of these channels to RIPC using pharmacological antagonists of the K_{ATP} channels such as glibenclamide and 5-hydroxydecanoate [65] [67] [69]. In addition Weinbrenner et al showed that a free radical scavenger was able to abolish the protection elicited by RIPC, thus suggesting a role for reactive oxygen species (ROS) in the mechanistic pathway in RIPC [70].

From Distant Organ to the Heart

The mechanistic pathway from distant organ to the heart remains unclear. Current thoughts are that there are three main mechanisms: humoral, neural, and systemic, which interact with each other, conferring protection upon the target organ from a distant site.

- *Humoral*

  Numerous studies have shown that a humoral factor appears to play a role in the mechanism of cardio-protection offered through RIPC. Dickson and colleagues hypothesized that if the trigger signal responsible for RIPC is humoral in nature then it should be transferable from one animal to another of similar species. Using paired, blood cross-matched rabbits, the group were able to show that the remote preconditioning effect could be transferred from one preconditioned rabbit to a non-preconditioned rabbit heart via whole blood transfusion [71 72]. Further evidence that RIPC involves a humoral mediator comes from work done by Konstantinov et al. Their results showed limb RIPC of pigs that had received a transplanted hearts was able to reduce myocardial infarct size in the donor heart [67]. Identification of the humoral factor or factors that conveys protection from RIPC is proving a challenge, particularly because they may be active at very low concentrations amongst the huge protein content of the blood. Shimizu and colleagues showed, in a Langendorff isolated rat heart model, that prior -perfusion with either the plasma from donor rabbits subjected to RIPC similarly protected against infarction. The effectiveness of the RIPC plasma was lost after passage through a hydrophobic column, but eluate from this column provided the same level of protection, suggesting the factor is hydrophobic. The plasma was dialysed to remove low molecular weight components, and protection was lost, suggesting that the protective factor is of a low molecular mass (<15kda). The group additionally showed similar results using dialysate of RIPC plasma from humans, in an in vitro cardiomyocyte model of IR, showing increased protection when compared to control. Similar studies have narrowed down the protective factor to a range of 3.5 kDa to 15 kDa [73,74] but proteomics has yet to reveal the identity of the peptide. Humoral factors such as bradykinin, opioids, adenosine and endocannabinoids have all been investigated since many are involved in direct IPC and are therefore obvious potential candidates for RIPC. There is increasing evidence for opioid receptor involvement in the cardio-protective mechanism of RIPC [75–77]. It is hypothesized that endogenous opioids are generated by the RIPC stimulus in the

remote donor organ, which then enter the blood stream and then act directly on the myocardium to confer cardio-protection. Patel and colleagues first demonstrated that naloxone, a non-specific opioid receptor blocker, was capable of abolishing the protection conferred by mesenteric RIPC in rats. Shimizu and colleagues explored this further using RIPC dialysate, with which they discovered cardio-protection was also lost after the addition of naloxone [78]. Both the δ1-opioid receptor and the κ-opioid receptor have been implicated in RIPC cardio-protection [79 70 80]. However the relative contributions to cardio-protection from these different opioid receptor subtypes remain unclear.

Bradykinin has been found to be a key mediator in ischaemic preconditioning. Work carried out by Schoemaker and colleagues also suggest its involvement in RIPC. Their results showed the protective effects of RIPC through brief mesenteric artery occlusion was lost through the administration of a HOE-140, a bradykinin B2 receptor antagonist [81]. However more recently a study in human volunteers investigated further the role of bradykinin in RIPC in human volunteers. Endothelium-dependent vasomotor function as investigated in human volunteers who were given a RIPC protocol with and without the bradykinin B2 receptor antagonist (HOE-140). Their results showed no difference in the impairment of endothelium-dependent vasomotor function in volunteers who had been given RIPC and a bradykinin B2 receptor antagonist compared to volunteers who had been treated with RIPC alone. Both showed significant reduction in the impairment of endothelium-dependent vasomotor function when compared to the non-RIPC treated control volunteers [82]. Yellon's group first described the mechanistic role of adenosine in RIPC. The reduction in myocardial infarct size achieved through renal artery RIPC was found to be lost after administration of the non-specific adenosine receptor antagonist 8-sulphophenyltheophylline (8-SPT) [65]. While this data suggests adenosine receptor binding is involved during RIPC, Takaoka and colleagues demonstrated that adenosine receptor binding is also required for cardio-protection, in experiments in which 8-SPT was administered after the RIPC protocol but prior to the extended ischaemia[83]. Increased plasma levels of adenosine has also been found in blood taken from the carotid artery of rabbits subjected to RIPC when compared to rabbits treated with IPC alone [84]. Most recently other humoral factors such as interleukin 10 and SDF-1α have, in addition, been implicated in playing a mechanistic role in RIPC [85,86]. It is highly plausible that multiple humoral factors are involved in the mechanism of protection behind RIPC. These factors are not mutually exclusive and may operate in parallel or in series. However how they get from the distant organ to the heart is yet not known. Transfer

may well be via the blood stream however many humoral proteins such as SDF1α are highly labile or subject to proteinase degradation. There may be protective mechanisms that prevent this degradation that allow safe transfer and further investigation is needed.

- *Neural Pathway*

Some have hypothesized that neural pathways may play an important role in the protective mechanisms that underpin RIPC. Evidence shows that these neural pathways work in conjunction with the humoral factors mentioned previously. Having demonstrated the involvement of bradykinin as cited above, Gho and colleagues went on to demonstrate that the protection conferred through intra mesenteric administration of bradykinin was sensitive to ganglion blockade by hexamethonium [51]. They hypothesised that bradykinin stimulated by RIPC may then go on to stimulate mesenteric afferent sensory nerves which then go on to mediate the cardio-protective effects of RIPC. Thus both pathways may be involved serially. Further evidence of a neural pathway came from work done by Ding et al. The group showed that cardio-protection from renal artery-RIPC was lost after renal nerve section. They also demonstrated increased renal nerve discharge during a renal preconditioning stimulus. Interestingly they additionally showed that this increase in renal nerve discharge was also blocked by administration of 8-PT. Thus like bradykinin it seems, adenosine may exert it effects via neural pathways [87]. Liem and colleagues further investigated this hypothesis. They were able to confer cardio-protection via administration of adenosine into the mesenteric vascular bed and as per Schoemakers' work with bradykinin, cardio-protection was lost after ganglion blockade with hexamethonium [88]. These findings suggest similarly to bradykinin that the renal artery RIPC stimulus leads to increased adenosine concentration, which activates mesenteric afferent nerves, which in turn mediate cardio-protection. Work carried out by Dong and colleagues shows evidence for a neural pathway extending beyond renal RIPC stimulus. In their experiments, they demonstrate the cardio-protective effect of remote hind limb preconditioning was obliterated by femoral nerve dissection, suggesting the need for an intact neural pathway for cardio-protection via RIPC. They were also able to demonstrate the role of adenosine in reduction in myocardial infarct size through direct intra-femoral artery administration of adenosine. [89] Tang et al have additionally suggested the neurotransmitter, calcitonin gene-related peptide (CGRP) as a potential mediator in RIPC. Through their experiments they conclude that RIPC stimulates capsaicin-sensitive sensory nerves which release CGRP, which is carried to the heart in the

blood stream where it exerts a cardio-protective [90]. It is therefore clear that neural pathways play a key mechanistic role in cardio-protection conferred through RIPC. Endogenous humoral substances such as adenosine and bradykinin released by the remote preconditioned organ go on to stimulate afferent nerve fibres, which then relay to efferent nerve fibres terminating on the myocardium to confer cardio-protection.

- *Systemic Inflammatory response*

  There have been several studies that have shown a systemic inflammatory response to RIPC. Work carried out on humans and rats by Konstantinov IE and colleagues showed suppression of genes encoding proteins involved in cytokine synthesis, leukocyte chemotaxis, adhesion and migration, exocytosis, innate immunity signaling pathways, and apoptosis. Interestingly suppression of these genes took place within 15 min of the RIPC protocol and even more so after 24 h [67]. This suggests RIPC induces global myocardial genomic responses that lead to increased resistance to ischaemic and oxidative stress both early and late after the stimulus. Furthermore, the rapidity of the response suggests the possibility of active mechanisms at mRNA suppression such as miRNA. Indeed the few clinical trials looking at the inflammatory response to RIPC have shown increases in inflammatory markers such as C-reactive protein and Interleukin-6[91,92].

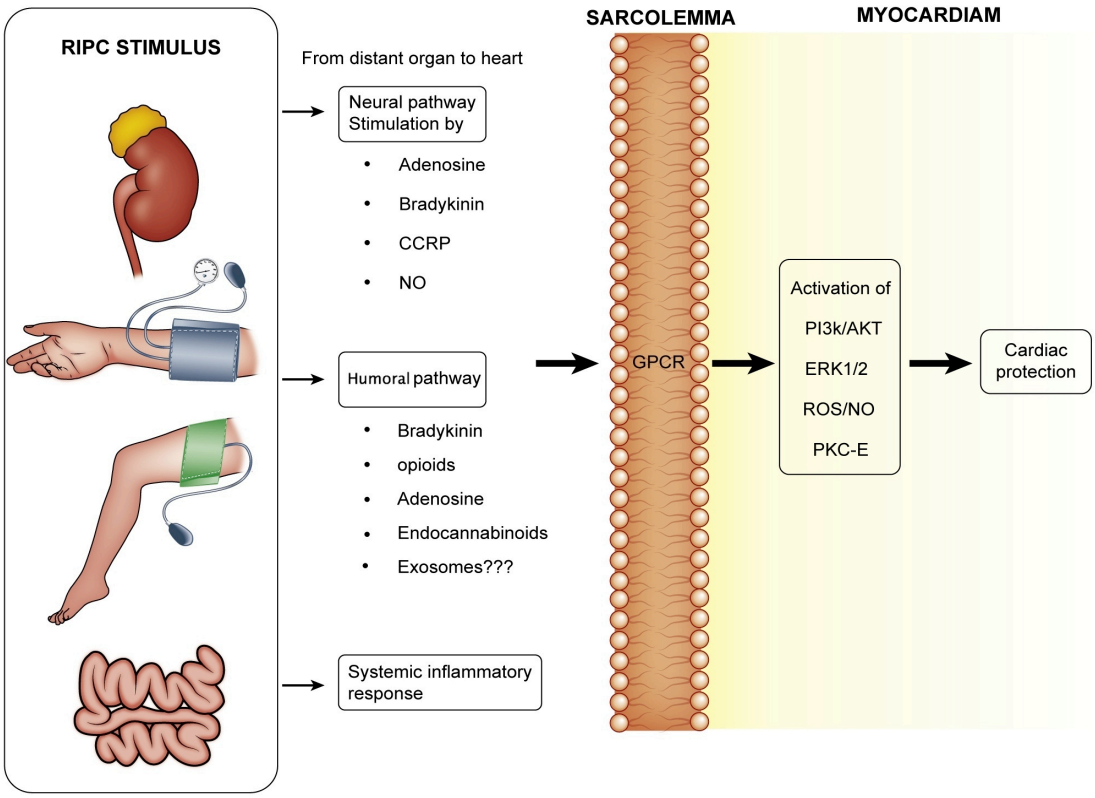


Figure 3 **Diagram representing the mechanism of RIPC.** It is hypothesised that the RIPC stimulus of a distant organ such as kidney, forearm, leg or small intestine leads to stimulation of neuronal and humoral pathways as well as activation of a systemic inflammatory response. This then activates the sarcolemal GPCR and exerts cardio-protective effects via the traditional RISK pathway.

Preconditioning in the Clinical Setting

Human Atrial Trabaculae

The majority of evidence for preconditioning the myocardium comes from animal studies with the effects being extrapolated to humans. This therefore potentially introduces errors due to species differences. Preconditioning as a cardio-protective strategy in humans has not been easily translated into routine clinical use as clinical trials assessing the use of preconditioning in humans have been challenging. This is largely due to ethical constraints of investigating with human participants together with the inability to reliably predict an onset of myocardial infarction. The human atrial trabaculae model of IR provides one solution to the above problems. This experimental model was first established in by Yellon's laboratory and is both a robust and reproducible technique for assessing cardio-protective treatment strategies using

human atrial trabeculae subjected to simulated IR injury. Work from within our group was the first to show that preconditioning the human atrial tissue by brief periods of simulated ischaemia and reperfusion improved percentage recovery of tension after a sustained period of simulated ischaemia, when compared to control. [93] Importantly this model has also allowed for the allowed for assessment of the cardio protective signalling pathways seen in animal studies to be confirmed in human muscle[93,94].

Pre-Infarction Angina

It is hypothesized that pre-infarction angina may represent a form of "natural" preconditioning caused by antecedent episodes of transient ischaemia and reperfusion. There have therefore been a number of studies that have looked at outcomes of those patients presenting with AMI with and without pre-infarction the results of which provide an indication that the human myocardium may be amenable to preconditioning. Studies have shown that patients with a history of pre-infarction angina have a more favourable outcome than those without prior angina. Interestingly, this benefit was not seen in diabetics and the elderly, groups that are also difficult to precondition in animal studies, although in this setting preconditioning is possible but it appears that the threshold for protection is raised in diabetes and age [95]. Patients presenting with myocardial infarction who have had pre-infarction angina have been found to have less release of cardiac enzymes [96–98]. Patients with pre-infarction angina have been shown to have a greater degree of myocardial viability in the infarcted area [99]; Improved left ventricular function and reduced frequency of severe congestive heart failure when compared to those that have not [97,98,100–102]. Pre-infarction angina is also associated with a reduction in life threatening ventricular arrhythmias and reduced in hospital and one year mortality [97,98]. These effects of pre-infarction angina were independent of cardiac risk factors, medical therapy and coronary collateral score [98,99]. In another parallel to animal study models, and with similarities to the time course of protection seen in the "second window" of protection, pre-infarction angina appears only protective if it occurs within 24 to 48 hours of myocardial infarction.

Warm up angina

"Warm up " angina refers to the observation that exercise-induced ischaemia on second effort is significantly attenuated or even abolished if separated from first effort by a brief rest period [103]. If we hypothesis that the initial exercise induced ischaemia is acting as a preconditioning stimulus therefore the phenomenon of "warm up" angina provides us with anecdotal evidence that the human heart may be amenable to

preconditioning. Though the mechanism behind the phenomenon of warm up angina is yet to be fully understood, the findings thus far indicate a process of prompt metabolic adaptation of the myocardium, which induces tolerance to subsequent ischemia, a process similar to classical preconditioning.

Coronary artery bypass grafting (CABG)

Patients who undergo CABG provide suitable candidates or the investigation of preconditioning in humans. Though it can be argued that the ischaemia associated with CABG surgery is different from that experienced from an AMI, in which CABG patients often experience a global ischaemia as compared to the regional ischaemia experienced from an AMI. These patients are undergoing a planned procedure, which inevitably causes a degree of myocardial ischaemia and reperfusion injury that can potentially be targeted by a preconditioning stimulus. Yellon's group first studied this in 1993 in 14 patients undergoing CABG randomized into two groups. The preconditioning stimulus involved intermittent application of an aortic cross clamp to deliver repeated episodes of global ischaemia. Measurements of ATP levels in myocardial biopsies of the patients were used as the endpoints. Results showed those patients subjected to the preconditioning stimulus had better preservation of ATP levels in their myocardial biopsies[104,105]. These metabolic changes show stark similarities to those seen in a dog model of ischaemic preconditioning [105]. Using the same protocol a further study by Yellon's group measured the degree of serum troponin leak as a marker of myocardial necrosis. Results showed the preconditioned patients at 72 hours post surgery had significantly lower serum troponin T concentrations than those who were not preconditioned [106]. Though these studies have shown some encouraging results, the application of direct preconditioning in cardiac surgery has been met with resistance because intermittent cross-clamping can prolong surgery by up to 30 minutes which in turn increases the risk of other procedure-related complications such as the risk of embolism etc. Noticeably the addition of preconditioning to other cardio-protective strategies such as hypothermia or cardioplegia, has not delivered any additional benefit [107,108].

Percutaneous coronary intervention

Percutaneous coronary intervention remains the gold-standard treatment for an AMI, thus the ability to precondition the human myocardium in this setting would provide an invaluable tool in the treatment of this disease process and reduce the degree of myocardial injury caused by the intervention itself. The procedure usually involves

repeated intracoronary balloon inflations with intervening periods of perfusion. Studies have examined the hypothesis that the first period of ischaemia may enhance the myocardial tolerance to subsequent balloon inflations via classic ischemic preconditioning. Studies have consistently shown that first balloon inflation longer than 60-90 seconds significantly attenuate a number of markers of myocardial injury during subsequent balloon inflations. These markers include lactate production, release of myocardial enzymes, angina severity, left ventricular regional wall motion abnormalities, ST-segment elevation, QT dispersion and ventricular ectopic activity [109–115]. K_{ATP} channels, adenosine and bradykinin appear to play a mechanistic role in the increased tolerance to successive balloon inflations during PCI [116]. Studies in human volunteers where patients were administered IV adenosine and bradykinin separately showed that both mirrored the protective effects of the first balloon inflation [117,118]. As in animal experiments, opioid receptors too have been implicated. In a study of 20 patients, administration of naloxone was able to abolished the protective adaptation to ischemia observed from the first prolonged balloon inflation [119].

Clinical application of remote ischaemic preconditioning (RIPC)

The advantages of RIPC when compared to local ischaemic preconditioning lie with its non-invasive nature and ease of application. Additionally local ischaemic preconditioning runs the risk of further myocardial dysfunction, low cardiac output, risk of arrhythmia, or secondary organ injury, this in comparison to the largely benign effects of transient skeletal muscle ischaemia. There is great interest in the clinical translation of RIPC as a cardio-protective strategy. RIPC in the setting of cardiovascular disease has been largely evaluated in three clinical settings; prior to cardiac surgery; prior to percutaneous coronary intervention and lastly in patients presenting with ST elevation myocardial infarction.

Cardiac Surgery

Günaydin and colleagues were one of the first to study the effects of remote ischemic preconditioning in 8 patients who were undergoing coronary artery bypass grafting (CABG). Participants were randomised to receive either RIPC (forearm cuff inflated to 300 mmHg for 2 cycles of 3 minutes) or control. Blood samples were collected immediately before cardiopulmonary bypass, prior to de-clamping aorta and 5 min after de-clamping the aorta, to determine creatinine phosphokinase (CPK), CPK-MB and lactate dehydrogenase (LDH) levels. The study demonstrated an increase in lactate dehydrogenase (LDH) in the preconditioned group. According to previous reports,

preconditioning of the myocardium results in a beneficial decrease in anaerobic glycolysis resulting in less acidosis and accumulation of lactate [120]. Though the results of this study show an increase in lactate dehydrogenase in the preconditioned group. Günaydin and colleagues hypothesized that preconditioning appeared to protect myocardium by enhancing anaerobic glycolysis. It is not clear how one can draw this conclusion from this study. The study is grossly underpowered with only 8 patients and shows contradictory results to what one would expect. As previously described with an increase in glycolysis one would expect a corresponding decrease in LDH in the preconditioned group. [121] In this study we see the opposite. In fact is the increase in LDH seen in the preconditioned group as a result of increased cell death? In 2006 Cheung and colleagues carried out a randomised controlled trial investigating RIPC in 37 patients undergoing paediatric surgery for congenital cardiac defects. The results showed that the RIPC protocol of 4 cycles of 5 minute lower limb ischemia via cuff inflation prior to surgery was effective in reducing troponin levels, postoperative inotropic requirements, and airways resistance at 6 hours [122]. An interesting study by Zhou W et al attempted to look at utilizing the protective effects of both classical RIPC and the "second window" of protection of RIPC. Patients undergoing ventricular septal defect repairs were randomised to a preconditioning group receiving three 5-min cycles of blood pressure cuff induced, limb ischaemia 24 hours before, and then again 1 hour before surgery. Results showed a reduction in cardiac enzyme release (LDH, CK and troponin I) post surgery in the preconditioned group. Additionally the preconditioned group also demonstrated reduction in inflammatory cytokine markers (IL-6, IL-8, IL-10 and TNF-α) and improvement in lung compliance [123]. In 2007, Yellon's group investigated the role of RIPC in the context of 57 patients undergoing CABG. A reduction in troponin T levels was observed in those patients randomized to receiving a RIPC protocol of 3 cycles of 5-minute forearm cuff inflation to 200 mmHg after induction of anaesthesia and prior to surgery [124]. In 2009 they went on to demonstrate a similar reduction in post-operative troponin I by administration of the same RIPC protocol in patients undergoing cold blood cardioplegia [125]. Disappointingly, in 2010 a larger, single-centre study of 162 patients undergoing CABG who were randomised to receive either three, 5-minute cycles of upper limb cuff inflation to 200 mmHg and deflation or placebo demonstrated that RIPC did not attenuate troponin release, improve hemodynamics, or enhance renal or lung protection [126]. The reasons for these divergent findings are not clear. An inadequately powered study is the most likely cause for a false negative in this study, if indeed it truly is a false negative. Interestingly in all the positive studies of RIPC in the context of CABG surgery, the RIPC stimulus is administered prior to cardiac surgery however in this one negative study the RIPC stimulus is administered after surgical incision.

A further 3 studies in 2010 were carried out investigating RIPC as a cardio-protective strategy in the context of CABG surgery. The first a study by Thielmann and colleagues demonstrates a 44.5% reduction of area under the curve for Troponin I levels at 72 h for patients given a RIPC protocol who had undergone elective CABG surgery with crystalloid (Bretschneider) cardioplegic arrest[127]. Following this a study by Wagner and colleagues investigated whether one could confer similar protection through RIPC via the "second window" of protection in patients undergoing CABG with cold-crystalloid cardioplegia [127]. An RIPC protocol of three five-minute cycles of upper limb ischaemia and three five-minute pauses using a blood pressure cuff inflated to 40 mmHg more than the patient's actual blood pressure was given to patients 18 hours before the procedure which resulted in a significant reduction in post operative troponin levels when compared to control [128]. Hong and colleagues studied the effect of RIPC on 130 patients undergoing off-pump coronary artery bypass graft surgery (OPCABG). An RIPC stimulus four cycles of five-minute ischaemia and reperfusion on the upper limb using a pneumatic cuff resulted in a 26% reduction in area under the curve for postoperative Troponin I levels when compared to control, though this did not reach statistical significance [129]. Hong and colleagues revisited this hypothesis in 2012 in 35 patients scheduled for OPCABG to receive an RIPC and a remote post conditioning (RIPostC) protective strategy compared to a control group. In the RIPC+RIPostC group, 4 cycles of 5-min ischemia and 5-min reperfusion were done on a lower limb before anastomoses (RIPC) and after anastomoses (RIPostC). Results this time showed those patients who had RIPC+RIPostC strategy had significantly reduced postoperative serum troponin I levels with the area under the curve for postoperative troponin 48.7% lower in the RIPC+RIPostC group when compared to controls [130]. Interestingly a study by Karuppasamy and colleagues in 2011 in 54 patients referred for elective CABG surgery who were randomized to receive the standard RIPC protocol of three 5 minute cycles of left arm cuff inflation and deflation or control, showed no difference in post operative troponin levels between the two groups. In this study patients were given an anaesthetic regimen of both isoflurane and propofol [131]. Kottenberg and colleagues hypothesized RIPC had differential effects depending on background anaesthesia given. In there study of 72 patients undergoing CABG surgery, serum troponin I concentration at various time points were measured during isoflurane/sufentanil or propofol/sufentanil anaesthesia with or without RIPC. Interestingly their results demonstrates RIPC during isoflurane but not during propofol anaesthesia was able to attenuate myocardial damage [132]. In 2012 D'Ascenco and colleagues carried out a meta-analysis of the nine studies mentioned above with 704 patients included. Standardised mean difference of troponin I and T release showed a significant decrease in the groups treated with an RIPC protocol (-0.36 (95% CI -0.62

to -0.09)) [133]. More recently Yang et al carried out a larger meta-analysis of nineteen randomized trials involving 1,235 patients. The cTnI concentrations at 6 (or 4-8) hours postoperatively and the total cTnI released after surgery showed a statistically significant reduction in the RIPC group compared with a control group. In addition the group found no differences in mortality, morbidity, and resource utilization between groups[134]. Heusch and colleagues attempted to look at the effect of RIPC on mortality in patients undergoing elective CABG. They enrolled 329 patients scheduled to undergo elective, isolated first-time CABG under cold crystalloid cardioplegia and cardiopulmonary bypass between April 2008 and October 2012. Patients who were randomized to an RIPC stimulus showed similar trends with reduction in perioperative myocardial injury by means of reduction in the mean area under the curve for concentration of cardiac troponin I. However more importantly they were able to demonstrate reductions in 1-year all-cause mortality in the RIPC group. MI rates also were reduced, while rates of cardiac death, stroke, and MACCE (postoperative MI and TIA or stroke) all showed trends favouring remote preconditioning [135]. RIPC in the setting of cardiac surgery therefore shows some promise and requires further investigation to truly determine whether remote ischaemic preconditioning improves clinical outcomes after cardiac surgery. One such trial led by our group is under-way, The “ERICCA” trial a multicentre randomised controlled clinical trial, which has now recruited nearly 1600 patients, the results of which are hotly anticipated [136]. In this randomized double-blinded controlled clinical trial circa high-risk patients undergoing CABG ± valve surgery using blood cardioplegia are being recruited over a 2-year period. Post anaesthetic induction patients will be randomized to receive after either RIPC (4 cycles of 5 min inflation to 200 mmHg and 5 min deflation of a blood pressure cuff placed on the upper arm) or sham RIPC (4 cycles of simulated inflations and deflations of the blood pressure cuff). The primary end points are cardiovascular death, non-fatal myocardial infarction, coronary revascularization and stroke at 1 year. Secondary endpoints will include peri-operative myocardial and acute kidney injury, intensive care unit and hospital stay, inotrope score, left ventricular ejection fraction, changes of quality of life and exercise tolerance. The results of this study may provide the definitive answer if RIPC can result in the hard end point of improving clinical outcomes.

Elective Percutaneous Coronary Intervention (PCI) or for Acute Myocardial Infarction (AMI)

Iliodromitis and colleagues were the first to investigate RIPC in the context of PCI. The study consisted of 41 normotensive patients with stable angina and single-vessel

disease undergoing elective PCI of which 20 were given a RIPC protocol of 3 cycles of 5-minute ischaemia applied to both arms along with 21 controls. Interestingly they demonstrated an increase in CK-MB, troponin I, and CRP in the preconditioned group [91]. Though disappointing, the results of this study must be taken with extreme caution. With only 41 patients the study is grossly underpowered to provide a reasonable conclusion. The reporting of the study is also misleading. In such a small population it would be reasonable to show the individual troponin levels for each participant. This would allow the observers to see if there are any erroneously high troponin levels that skew the data one way or the other. Secondly on further analysis the study group includes a significant number of diabetic patients, who are known to be less responsive to preconditioning. Lastly the high use of nitrates, a known cardio-protective mimetic, in the control arm may also skew the results in such a small study. Moving forward, Hoole et al in 2009 investigated further in the larger "Cardiac Remote Ischemic Preconditioning in Coronary Stenting (CRISP)" study of 242 patients undergoing elective PCI. The group demonstrated that RIPC (3 cycles of 5-minute forearm cuff inflation to 200 mmHg and deflation) prior to PCI attenuated post procedure cardiac troponin I (cTnI) release. Hoole and colleagues were also able to show a reduction in the major adverse cardiac and cerebral event rate (MACCE) at 6 months in the RIPC group [137]. Since this study there have been a number of studies that have consistently demonstrated similar reductions in procedural related troponin I release [138,139]. Hoole and colleagues were interested to confirm if this early benefit in MACCE rate in the remote ischaemic-preconditioning group was sustained and had a long-term effect. The group have recently published a six-year follow up data of the original cohort. 89.3% of the original study was available for long-term follow-up with a total of 59 (30.7%) MACCEs. The group was able to demonstrate patients with a MACCE had higher mean cardiac troponin I after PCI, and most interestingly the MACCE rate at 6 years remained lower in the RIPC group. Hazard ratio was 0.58 at 95% confidence interval in the RIPC group and an absolute risk reduction of 0.13. The number needed to treat with RIPC to prevent a MACCE at 6 years was only 8 [140]. This is the first study to provide objective evidence that RIPC, a cheap and relatively risk free intervention, can have a prognostic benefit in those undergoing elective PCI. Bøtker and colleagues investigated the use of RIPC in 333 patients the setting of an acute myocardial infarction (AMI). They investigated the potential for use of RIPC as a pre-hospital cardio-protective intervention in patients having an AMI. The RIPC protocol involved 4 cycles of 5-minute forearm cuff inflation and deflation delivered in the ambulance while in transit to hospital. The primary end point was myocardial salvage index at 30 days after primary percutaneous coronary intervention measured by myocardial perfusion imaging as the proportion of the area at risk salvaged by treatment. Results

demonstrate a significant improvement in myocardial salvage index in the RIPC group with no differences in major adverse events. In a sub-study of the same patients the investigators looked at the short term effects of LV function. The results demonstrate no significant overall effect on LV function between RIPC and control groups. However improvement in LV function were seen in high-risk patients, those with extensive myocardial area at risk (AAR) AAR ≥35% and therefore prone to develop large myocardial infarcts [116]. Most recently Crimi et al published their study, which sought to evaluate whether remote ischemic post-conditioning (RIPostC) could reduce enzymatic infarct size in patients with anterior ST-segment elevation myocardial infarction undergoing primary percutaneous coronary intervention. 100 patients with anterior ST-segment elevation myocardial infarction and occluded left anterior descending artery were randomized to primary coronary intervention (pPCI) + RIPC (n = 50) or conventional pPCI alone (n = 50). RIPC consisted of 3 cycles of 5 min cuff inflation and deflation of the lower limb. Promisingly RIPC at the time of pPCI reduced enzymatic infarct size measured by CK-MB. The group were able to demonstrate a relative reduction of CK-MB release by 20%(confidence interval: 0.2% to 28.7%; p = 0.043). In addition T2-weighted oedema volume 3-5 days post MI, measured through cardiac MRI was 37 ± 16 cc in RIPC patients and 47 ± 22 cc in control subjects (p = 0.049). Lastly ST-segment resolution >50% was 66% in RIPC and 37% in control subjects (p = 0.015). In addition the patients who had the RIPC stimulus were also associated with an improvement of T2-weighted oedema volume and ST-segment resolution >50%

Conclusion

RIPC has been investigated in other clinical settings such as vascular surgery, cerebral and neurological injury, as well as acute kidney injury with mixed results. There are a number of confounders that make the translation of laboratory findings on young healthy rat hearts to the complexity of the human body. In clinical studies the biggest confounders are age, sex, other comorbidities and concomitant drug medications. We know from laboratory findings that these all play a role in the ability to protect the heart. Older and diabetic hearts are harder to cardio-protect. The patient is often already on a cocktail known cardio-protective pharmalogical agents such as metformin, simvastatin etc. thus the extent and potential for further cardio-protection is unclear if not small. What is clear is that RIPC has the potential to become a valuable adjunct to current medical therapy that can help harnesses a powerful innate protective mechanism to aid against ischaemic injury. Though the mechanism is yet not fully understood however it has shown promise in clinical trials thus far. Large adequately powered studies such as

the Effect of Remote Ischaemic preconditioning on clinical outcomes in patients undergoing coronary artery bypass graft surgery (ERICCA) will report within the next 2-3 years that will provide more definitive answers. This hopes to be the definitive randomised control trial looking at the effects of RIPC on the hard end points of Major Adverse Cardiac and Cerebral Events (MACCE) 12 months after cardiac surgery. The results of this trial will prove to be a defining moment in this research field.

Extracellular Vesicles

Extracellular vesicles are a heterogeneous population of membrane bound structures released by cells in to the extracellular space. Their role in disease and their potential for diagnostic and clinical use is under increasing investigation. A consensus nomenclature for these heterogeneous vesicles is of yet not fully established. They can largely be subdivided in to three main populations exosomes, shedding microvesicles and apoptotic bodies.[141]

Shedding Microvesicle

Shedding microvesicles bud directly from the plasma membrane and are then secreted directly into the extracellular environment. Although usually larger in size when compared to exosomes (30-100nm), shedding vesicles can range from 10nm-1μm in size and are more heterogeneous in shape. In contrast to exosomes, which are generated via the endosomal pathway, budding from the plasma membrane generates shedding microvesicles. In turn the membrane of shedding microvesicles are more reflective of their originating cell than the membranes of exosomes.[142,143] Studies have shown Ca^{2+} stimulation cells results in an increased secretion of shedding vesicles. The mechanisms, which underpin the sorting of protein cargo within shedding microvesicles, are not yet understood. Studies thus far have shown a universal expression of β1 integrin on the membrane of all shedding vesicles. Platelet shedding vesicles contain GPIb and GPIIb–IIIa and P selectin, which are important in coagulation. Shedding microvesicles from tumor cells and neutrophils contain proteolytic enzymes and metalloproteinases that are thought to play a role in inflammation and cancer progression.[143] Studies have in addition shown that shedding vesicles released from the same cell under rest and stimulation can be molecularly distinct from each other.[144] Shedding microvesicles have been shown to play a role in coagulation, inflammation and tumor progression. They have been shown to induce platelet activation and thus play an important role in the pathological process of atherosclerosis. Shedding vesicles from platelets and macrophages accumulate in the

lipid core of plaques and thrombi promoting further platelet activation and vascular dysfunction. [145–147] In contrast, the hypothesis, which was explored in this thesis, is that exosomes play a cardio-protective role.

Apoptotic Vesicles

Dying and/or apoptotic cells will also release membrane vesicles and are distinct from those vesicles released from healthy cells. Apoptotic vesicles, like shedding vesicles, bud directly from the plasma membrane and carry a number of nuclear, cytosolic, and endoplasmic reticulum (ER)-derived proteins. Apoptotic cells release at least two immunologically distinct types of apoptotic vesicles, those originating from the plasma membrane contain DNA and histones, whereas apoptotic vesicles originating from the endoplasmic reticulum do not but expose immature glycoepitopes[148]. In general, apoptotic vesicles are larger in size when compared to exosomes and shedding vesicles. In addition apoptotic vesicles float at a higher sucrose density (1.24-1.28 g/ml) than exosomes and do not have the exosomal characteristic cup shape morphology when visualized by electron microscopy, thus can be visually distinguishable from exosomes. Apoptotic vesicles have been reported to have immune-activating functions and the inappropriate clearance of apoptotic vesicles is considered to be a play a significant role in the development of systemic autoimmune disease. Winau et al investigated apoptotic vesicles from mycobacteria-infected macrophages. Their results show that apoptotic vesicles from mycobacteria-infected macrophages stimulate CD8 T cells in vivo and demonstrated potent adjuvant activity by stimulating through Toll-like receptors (TLR). Winau et al, showed that vaccination with vesicles from infected cells induced protection against M. tuberculosis infection[149]. The ability of exosomes to transfer genetic content has generated much interest, however this ability may extend to apoptotic vesicles as well. Some groups have suggested that apoptotic vesicles may play a role in the horizontal transfer of tumor DNA to distant sites, which then initiate the genetic transformation leading to tumor formation[150].

Exosomes

Exosomes are lipid bound membranous vesicles that range from 30-100nm in size [151–153]. These intra-luminal vesicles arise from endosomal compartments called multivesicular endosomes (MVE). Upon fusion with the plasma membrane, MVE release these vesicles in to the extracellular fluid at which point they are referred as exosomes. Exosomes have been found secreted by multiple cell lines as well as been

found in most bodily fluids including human plasma[153,154]. There has been more interest in exosomes and their possible involvement in health and disease, although originally ignored as cell "debris", their content such as mRNA and miRNA and the ability to transfer them are generating interest in the scientific community as a potential novel l mode of intercellular communication[151,152]. There is currently great interest in the potential of both exosomes and microvesicles as therapeutic targets or as biomarkers of diverse pathological states including cardiovascular disease, Alzheimer's disease, viral infection, and cancer[151,152,155].

History of Exosomes

Trams et al. in 1981 first coined the name "exosome" to describe microvesicles that were released from neoplastic cell lines[156]. Originally MVE, from which exosomes arise, were thought to help in the transfer of extracellular molecules to lysosomes for degradation. A few years later, however, two independent research groups studying reticulocytes described an opposing process[157,158]. They demonstrated the secretion of small vesicles (~50 nm) of endocytic origin by MVEs in cultured reticulocytes. These vesicles contained transferrin receptors that had been internalized from the plasma membrane[158]. Sequential electron microscopy images demonstrated a process of fusion of the MVEs with the plasma membrane with subsequent release of the transferrin containing vesicle outside the cell[157]. Following their electron microscopy findings Johnstone and colleagues were the first to re-isolate these nanovesicles, purifying the reticulocyte-culture supernatant by a process of centrifugation. The vesicles were then termed "exosomes"[159].

Research developments in the field of exosomes were fairly sparse for the following decade onwards. It was not until 1996 when Raposo et al. rejuvenated interest in this field with their paper suggesting these exosomes carry molecules that are involved in the adaptive immune response. In this study they showed firstly that EBV – transformed B lymphocytes secreted exosomes, and more importantly that these vesicles went on to stimulate human CD4 T Cells in an antigen specific manner, [160]. In 1998 the pioneering paper by Zitvogel et al. demonstrated the secretion of exosomes from dendritic cells. These dendritic exosomes expressed function major histocompatibity Complex class I and II and T-cell co-stimulatory molecules. The study also showed the dendritic derived exosomes suppressed growth of murine tumors in a T cell dependent manner[161]. This paper laid the foundation for the hypothesis that exosomes could play an integral and distinct role in cell-cell communication and has prompted researchers to explore its clinical application. Since then over the years

exosome secretion has been described in a multitude of cell lines in addition to the above mentioned previously; Hematopoietic cells (B cells, T cells, dendritic cells, mast cells and platelets) [160,162–164], neuronal cells [165], Schwann cells[166], intestinal epithelial cells[167], adipocytes[168], fibro- blasts[169] and tumor cells[170], cardiomyocytes[171]. As research within this field has increased the exosomal functions appear to be far more complex and each differ in its function dependent on its origin. Exosomes appear to work as highly specialized functional extensions of cells. They are largely released in biological fluids. To this date exosomes have been isolated from biological fluids such as plasma, urine, cerebrospinal fluid and, amniotic fluid and saliva[154,172–174]. They have additionally been isolated from malignant effusions, fluid from bronchoalveolar lavage, synovial fluid and even breast milk[175–177]. This may suggest that exosomes provide a mechanism of communication and transfer of material between distant body parts.

Exosome biogenesis

The majority of the exosomal proteins discovered to date are found in the cytosol, endocytic compartments or plasma membrane. In addition, many of the cytosolic proteins found in exosomes are involved in the endocytic pathway[178,179]. It is for these reasons that the current hypothesis is that exosomes have an endosomal origin. Exosomes are thought to be formed via endocytic invagination of the plasma membrane thus capturing material from the cell cytoplasm [180]. The formation of exosomes involves two membrane inversions allowing the retention of similar membrane topology as the plasma membrane. The first inversion occurs during endocytic internalization and the second inversion occurs as the intraluminal vesicles bud off into the lumen of the late endosome [180]. Cargos within MVE that are destined for lysosomal degradation are ubiquinated. The mechanism that underpins the re-routing of this MVE for exosomal release is still not yet fully understood. There is some evidence to suggest that exosome release may be mediated through endosomal sorting complex required for transport (ESCRT) signaling. In particular, a study by Thery et al. has shown Alix and Tsg101 to be involved in exosomal secretion in dendritic cells. Budding of the intraluminal membrane is thought to be mediated via ESCRT-I and ESCRT-II with ESCRT-III responsible for cleaving of the buds to form intraluminal vesicles [181]. Another model for exosome biogenesis suggests a ceramide synthesis driven system [182]. GTPases of the Rab family are proteins classically responsible for intracellular trafficking[183]. Evidence suggests that Rab family proteins may indeed regulate the targeting of the MVE towards the plasma membrane. In human haemopoetic K562 cells, Rab11 has been shown to promote docking and fusion of MVBs with the plasma membrane[184]. Rab27a and Rab27b play important

roles in spontaneous secretion of MHC class II-containing exosomes secreted by HeLa cells[185]. The consequent fusion of the exosome with the plasma membrane is then thought to be regulated by SNARE proteins [186]. Intracellular calcium levels may affect exosome release. A study my Merendino et al inhibited exosome secretion through the addition of DMA[187]. And in contrast the addition of monensin, Na+/H+ exchanger that results in the accumulation of intracellular calcium to K562 haemopoetic cells, markedly enhanced exosome release[188].

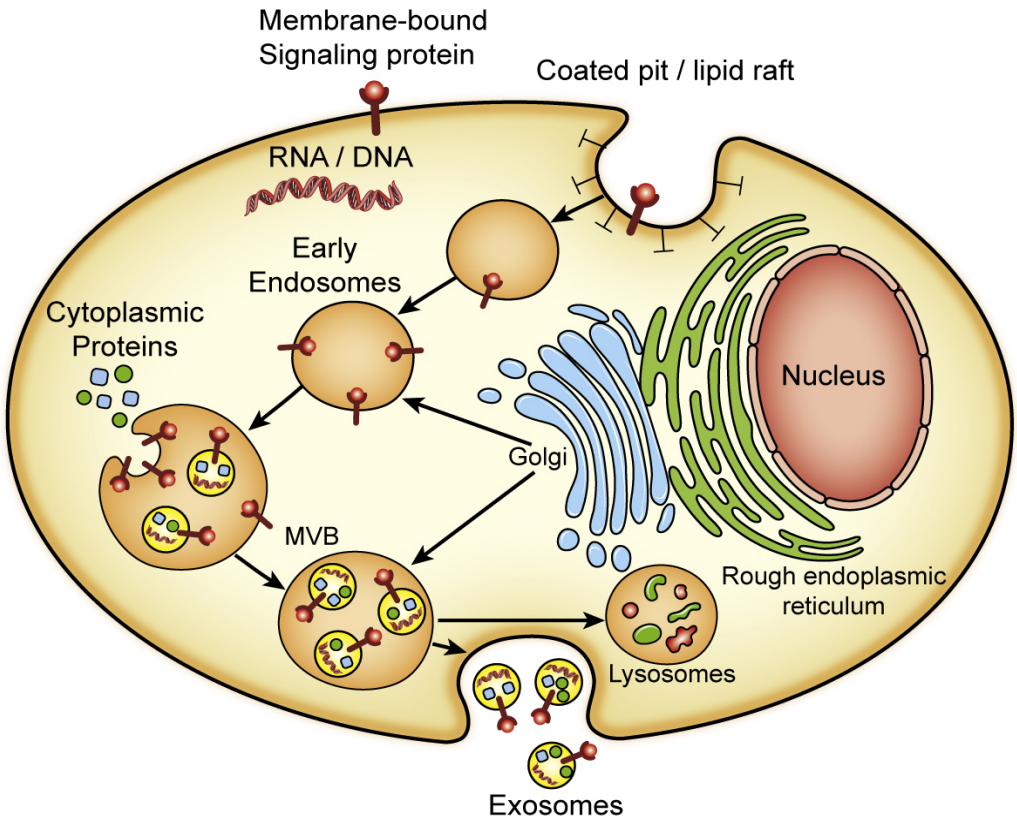


Figure 4 **Diagram describing exosome biogenesis adapted from Raposo et al 189. Exosomes formed via endocytic invagination of the plasma membrane capturing material from the cell cytoplasm.** The first inversion occurs during endocytic internalization and the second inversion occurs as the intraluminal vesicles bud off into the lumen of the late endosome. MVE that are destined for lysosomal degradation though some are re-routed resulting in release of exosomes.

Physical Characteristics & Molecular Composition of Exosomes

The size of exosomes makes visualization under visual light microscopy not feasible. Under electron microscopy exosomes exhibit a specific biconcave or “cup- shaped” morphology, although this is believed to be an artifact of drying of the spherical vesicles. They differ from the larger shedding microvesicles (sometimes called

microparticles) that are also found in plasma, in more than just their size. Shedding microvesicles are believed to form by budding off directly from the plasma membrane, and appear to have distinct properties from exosomes[151,155,164].

Exosomal cargo can often be donor-cell specific and in addition may be different dependent on circumstances of release e.g. oxidative stress. What drives this cargo selection within exosomes is still not understood.

- *Protein Composition*

  Large-scale proteomic analysis techniques have now allowed exosomal cargo to be described in some detail. This proteomic analysis of exosomes has shown common characteristic marker proteins on their surface or in their lumen. Typical exosomal marker proteins are the heat-shock proteins such as HSP70 and HSP90 and the tetraspanins such as CD9, CD63, and CD81. Additionally, exosomes have been shown to contain MVE biogenesis molecules such as Alix and TSG101 and cytoplasmic proteins such as actin, annexins, and Rab proteins[152]. These proteins may play important roles in disease. Indeed, the relationship between heat shock proteins and cardio-protection is well established. Heat shock proteins (including αB-crystallin, HSP60 and HSP70) are secreted from cells within exosomes[189,190], and can be transferred to adjacent cells to confer protection against oxidative stress[191]. Interestingly, circulating HSP70 levels are negatively correlated with symptoms of cardiovascular disease[192]. What is of further interest is that current evidence suggests that the exosomal proteome differs according to the type of cell that released it [152]. This may allow one to determine exosomal origin by the expression of typical cellular markers, an example of which is CD31 (PECAM-1) or CD62P (P-selectin) found in platelet derived exosomes[164]. The “Exocarta” database provides a useful tool and catalogues the proteins that have been identified in exosomes and also the number of studies in which they have been identified[193].

- *Lipid Composition*

  In addition to proteins exosomes have been shown to contain numerous raft-associated lipids such as cholesterol and sphingomyelin. The outer surface of exosomes has also been shown to contain a number of saccharide groups[153,194].

- *RNA and microRNA*

The discovery of mRNA and microRNA in 2007 by Jan Lötvall and colleagues was a pivotal moment in the field of exosome research. Most interestingly their *in vitro* studies demonstrated not only the presence of exosomal mRNA but that these were also functional mRNA. Transfer of mouse exosomal RNA to human mast cells lead to the formation of new proteins in the recipient cell[195]. This landmark discovery demonstrates a new form of cell-cell communication. Similarities are drawn to viruses where exosomes can "infect" cells with genetic material, which is translated to a functional protein. Similarly to the protein cargo, exosomal mRNA and miRNA cargo also reflect the cell they originate from, in addition, studies indicate that cellular stress can alter exosomal protein and RNA content[196].This suggests that exosomes may represent a snapshot of the physiological state of the cell released into the circulation. The extent to which plasma miRNA is contained within exosomes is still controversial. Some studies suggest that exosomes contain the majority of plasma miRNA[197], while others find plasma miRNAs are mainly in argonaute complexes[198]. However, numerous studies have demonstrated the ability of exosomes to transfer miRNA to other cells[195,199]. Exosomal miRNA and its relationship with cardiovascular disease will be discussed later.

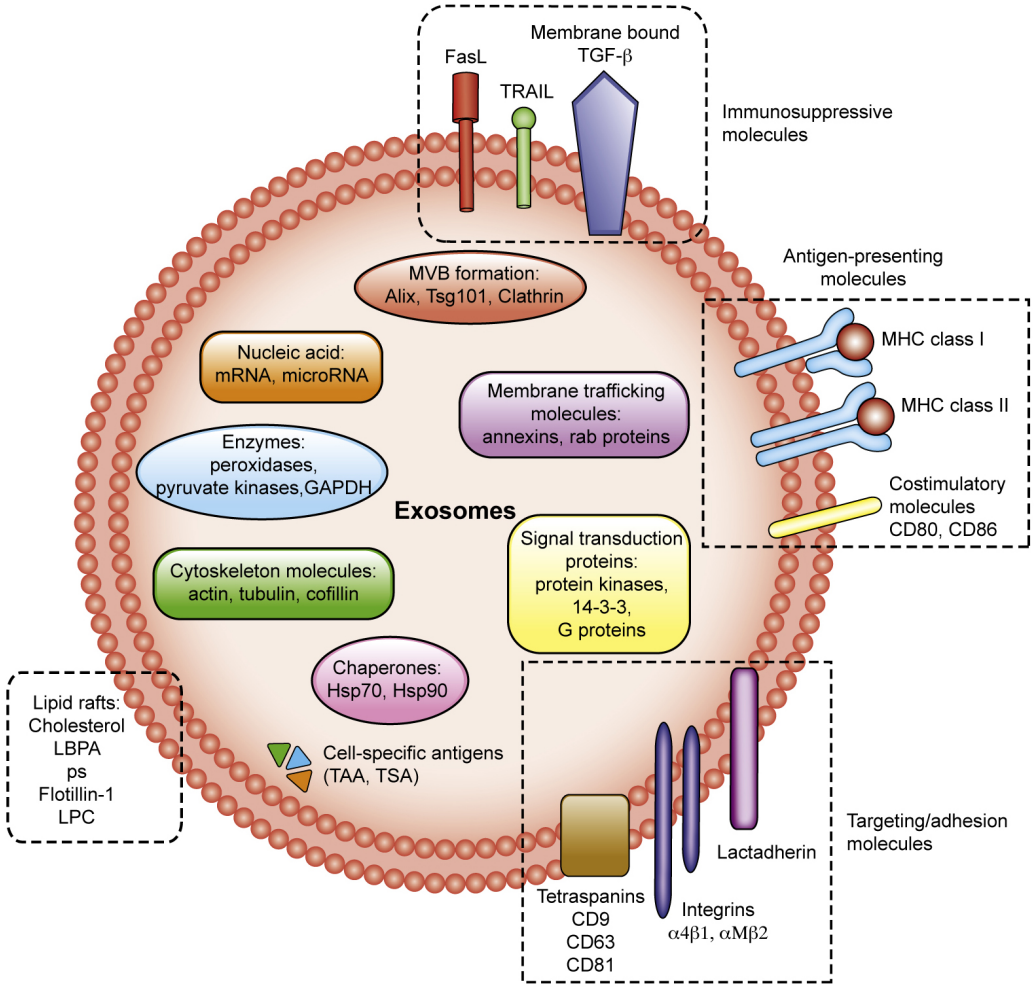


Figure 5 **Diagram describing exosome composition adapted from Thery et al 180.** Encircled by a lipid bilayer containing targeting and adhesion molecules, antigen presenting and immunosuppressive molecules. Within the exosome there are signal transduction and chaperone proteins, membrane trafficking molecules, mRNA and microRNA and lastly enzyme peroxidases as well as cytoskeleton molecules.

Function of Exosomes

The function of exosomes is not yet fully understood. Exosomes are secreted from a multitude of cells and it is clear that they affect a vast array of physiological functions, dependent on the cell of origin and circumstances at that time. Exosomes have been implicated in having a role in immune suppression and regulation, antigen presentation, tumour progression, and inflammation but to name a few. A detailed review of the function of exosomes is beyond the scope of this thesis and not investigated but the following is a brief summary.

- *Immune suppression by exosomes*

  Interest in exosomes was first ignited by the discovery that exosomes may play a role in immune suppression. Thus far the immunological effects that exosomes exert play appear to be dependent on the state of the secreting cells. An example of which is the secretion of exosomes from dendritic cells. Exosomes secreted from mature dendritic cells induce an antigen specific immune response. While dendritic cells that have been administered immunosuppressive treatments release exosomes that promote immune tolerance[200,201]. Immune modulation by exosomes means that, exosomes are likely to play a significant role in normal growth and development. Exosomes from various cell lines contain the death receptor ligand Fas ligand (FasL). It is thought that exosomes produced by the trophoblast in early pregnancy prevent maternal rejection through FAS mediated T-Cell death[202,203]. In fact pregnant women at term have higher levels of serum FasL associated exosomes when compared to with preterm, thus exosomes may play an immune-suppressive role that helps normal pregnancy. FasL exposing exosomes from tumor cells have been shown in vitro to kill Jurkat T cells. It is therefore hypothesized that this secretion of FasL containing exosomes from tumor cells helps the tumor cells in evading the body's natural immune system[204–206]. The exact role or contribution to immune suppression by exosomes is still as of yet not fully understood. Some studies have shown no immune suppression via exosomes, thus the exact role is likely to be model dependent and complex.

- *Antigen Presentation by exosomes*

  In addition to immune suppression, there is evidence to suggest exosomes play a role in antigen presentation. Exosomes appear to exert diverse effects dependent on origin and circumstance thus its role in antigen presentation is likely to be complex. Examples include injection of exosomes isolated from allergy tolerant mice to naïve mice conferred tolerance to the allergen. Thus, exosomes have the ability to prevent allergic sensitization[207]. In contrast when exosomes purified from Bronchoalveolar lavage fluid (BALF) of asthmatic patients were added to airway epithelial cells, a pro-inflammatory response of secretion of cytokines was noted. Thus exosomes may also contribute to and worsen allergic immune responses.[175]

- *Pro tumorigenic effects of exosomes*

The pro tumorinergic effect of exosomes occurs via several mechanisms. Tumour derived exosomes have been shown to have significant immunosuppressive properties and the immune evasion by tumour cells is a well-established mechanism wherein tumours avoid detection and elimination by the host immune system. An interesting finding by Abusamra et al. was that tumour derived exosomes release of FasL. FasL is important in the transmission of apoptotic signals and is lost in cancer cells[206]. Other evidence for immune suppression by tumour-derived exosomes comes from the down modulation of NKG2D due to direct exosomal delivery of membrane-bound trans membrane growth factor TGFβ1 to CD8+ T cell or NK cell subsets[208]. In cancer patients, the loss of NKG2D, an activating receptor for NK cells, CD8+ T cell and NKT cells, is a critical mechanism of cancer immune evasion. However some studies have shown tumour-derived exosome also carry antigens that help activate immune cells against cancer. It is therefore still unclear whether this contradictory role will result in tumour-derived exosomes providing a net pro-tumorigenic or anti-tumorigenic role at present. Other groups have suggested that tumour-derived exosomes aid tumour progression through the promotion of metastasis to distant sites. An interesting study by Hao et al. showed that exosomes could transfer the highly metastatic properties from a Bl6-10 melanoma tumour cell to a poorly metastatic F1 melanoma tumour cell. Hao and colleagues injected mice with F1 tumour cells only and other mice with F1 tumour cells and exosomes from as well as Bl6–10 melanoma tumour cells. Those injected with both, F1 tumour cells and exosomes from as well as Bl6–10 melanoma tumour cells developed lung metastases, compared to the mice injected with F1 tumour cells only, did not [209]. Exosomes are also thought to promote tumour growth through stimulation of angiogenesis. An example of which is seen in mesothelioma cell-derived exosomes. In a study by Hegmans et al. mesothelioma cell-derived exosomes were found to have strong angiogenic factors that increase vascular development in the region of a tumour[210]. In addition, melanoma-derived exosomes were also capable of stimulating endothelial signalling that is important for endothelial angiogenesis[211]. Further evidence of pro-angiogenic role for tumour derived exosomes comes from the evidence that exosomes secreted from a pancreatic the line that overexpress D6.1A in rats, strongly encouraged tumour growth through systematic induction of angiogenesis[212]. Other mechanisms for pro-tumorinergic role of exosomes include the release of active matrix-degrading enzymes, which increase the tumour's ability to invade into the stroma[213–215] . Tumour derived exosomes have also been shown

to promote tumour growth through modulating stromal cells[216]. An interesting finding from a study by Qu et al. found gastric cancer exosomes have been shown to promote cancer cell proliferation through activation of PI3K/Akt and MAPK/ERK pathway[217]. This pathway is of particular interest as it is one of the known cardio-protective survival pathways discussed previously.

- *Inflammation and exosomes*

  The role of exosomes in inflammation like in other areas discussed is complex? Dependent on the cell origin and circumstances exosomes can play a pro-inflammatory role as well as helping suppress it. Exosomal association with known pro-inflammatory proteins such as Hsp-70 and TGF-β is well described [189,218]. An interesting study by Qazi et al. describes pro-inflammatory exosomes in BALF of patients with sarcoidosis. The group described increased levels of exosomes in BALF from patients with sarcoidosis showed when compared with healthy individuals and when incubated with autologous peripheral blood mononuclear cells (PBMCs) exosomes from patients with sarcoidosis produced a significantly higher interleukin response[219]. In a study of fibroblast exosomes taken from synovial fluid of patients with rheumatoid arthritis, exosomes in the rheumatoid arthritic patients contained pro-inflammatory TNF-α that further potentiates the disease through delayed activation-induced death of infiltrated T cells[220].

Exosomes in Cardiovascular disease and Cardio-protection

The role of exosomes in cardiovascular disease is still largely unknown. Some of the few groups working on exosomes and its effects in the heart have arrived there serendipitously. Lim and colleagues first conceived interest in the field of exosome-derived cardio-protection through their work on mesenchymal stem cells (MSC)[221]. MSC were originally investigated as potential sources of newly differentiated cardiomyocytes when injected into recipient hearts, though this hypothesis has now largely been superseded given that few if any new cardiomyocytes have been observed. Despite the absence of demonstrable cardiogenesis the injection of stem cells has consistently shown improvement of cardiac function and survival. Current evidence suggests that MSC actually mediate their cardio-protective properties by release of paracrine factors[222]. Lim SK and colleagues systematically fractionated using membranes with different molecular weight cut off to demonstrate complexes with a diameter of 50–100 nm mediated the cardio-protective effect and thus hypothesized that these complexes were exosomes. The presence of exosomes was

then demonstrated by the detection of standard exosomal marker proteins (CD9, CD81, Alix), by Western Blotting, typical exosome density determined by sucrose density gradient, and observation of the typical “cup” shape by scanning electron microscopy. An important criterion is their diameter, which was shown by Nanosight Analysis (explained below) of purified fractions to be 100 nm - as expected for exosomes[221]. The exosomes were highly cardio-protective when introduced either into Langendorff perfused isolated rat hearts or intravenously into anaesthetized mice immediately before reperfusion. Subsequent studies show that cardio-protection by conditioned MSC medium requires activation of PI3K/Akt kinases in the protected organ[223,224]. Taking this concept further Chen et al investigated the cardio-protective properties of exosomes derived from murine cardiac progenitor cells (CPC). CPC-exosomes were incubated with H9C2 cardiomyoblasts. Their results demonstrate CPC-exosomes protect H9C2 from oxidative stress by inhibiting caspase 3/7 activation. In addition intra myocardial delivery of CPC-exosomes in an in vivo mouse model of myocardial ischemia/reperfusion resulted in a 53% reduction in cardiomyocyte apoptosis when compared to PBS control ($p < 0.05$)[225]. The most recent study by Giricz et al explored a similar hypothesis to that proposed in this thesis, i.e. that extra cellular vesicles (EV) might be involved in remote ischaemic preconditioning. In their small experiment the group collected coronary effluent from isolated perfused rat hearts subjected to 3 × 5 min global ischemia and reperfusion (IPC) or 30 min aerobic perfusion. The coronary perfusate with and without EV (removed by differential ultracentrifugation) was given to another set of recipient isolated hearts and subjected to ischaemia reperfusion protocol of 30 min global ischemia and 120 min reperfusion. Their results showed IPC markedly increased EV release from the heart as assessed by HSP60. As in preceding studies, perfusate of a preconditioned heart was able to attenuate the infarct of a non-preconditioned heart. However, interestingly, this protection was lost in the EV depleted perfusate. Though an interesting steps forward there are significant limitations to the study. The effluent transfer model from perfused hearts is a crude way of inferring a mechanistic role of EV’s in RIPC. Of note, electron microscopy characterization of their extracellular vesicles show that the majority of vesicles isolated and used in their ischaemia reperfusion protocol were indeed >100nm, outside the accepted exosome size[226]. Though not strictly relating to cardio-protection, work carried out by Cammusi et al show scientific principles that can be translated to the heart for further investigation. Cammusi et al in their study show that exosomes/microvesicles released from MSCs reverse acute kidney injury (AKI) by a paracrine mechanism by a horizontal transfer of messenger RNA and microRNA. The effect of exosome/microvesicle administration in murine models of kidney I/R injury and show exosome/microvesicle released from MSCs protect from AKI induced by

ischaemia reperfusion injury and from subsequent chronic renal damage. Interestingly this effect was obliterated with pretreatment of the exosomes/microvesicles with RNAse to inactivate their RNA cargo, suggesting the content of the vesicles playing a critical role in their function[227,228].

As a result of reduced coronary blood flow in ischaemic heart disease, collateral vessels and microvascular angiogenesis develop as a response to myocardial ischaemia over a period of time. It is thought that angiogenesis helps preserve the functionality of ischemic myocardium. Therapeutic coronary angiogenesis and collateralization have tremendous potential as treatment strategies for patients with ischemic heart disease. There is increasing evidence of exosomes having an important role in angiogenesis. Cammusi explored this further by showing that EVs promote angiogenesis and enhance recovery in a murine model of hind limb ischemia through miRNA or mRNA transfer[229,230]. Further work by Sahoo et al shows CD34+ stem cell derived exosomes induce angiogenic activity. Additionally HSP20 a protein known to play a role in cardio-protection was shown to be carried by exosomes that promotes angiogenesis via activation of vascular endothelial growth factor 2 (VEGFR2)[231]. Deregibus et al show angiogenesis stimulation via means of mRNA transfer from exosomes/microvesicles derived from human endothelial progenitor cells[230]. There are numerous further studies showing proangiogenic properties of exosomes from multiple cell lines, however many through differing mechanisms. In an interesting paper by Leroyer et al, extra cellular vesicles were isolated from ischemic muscles and injected into ischemic hind limbs of mice in parallel with venous injection of bone marrow-mononuclear cells. Addition of the EVs isolated from ischemic mouse hind limbs were shown increase the proangiogenic effect of bone marrow-mononuclear cell transplantation[232]. Exosomes may also play an important role in atherosclerosis. Recent work has shown that endothelial and smooth muscle cells, which play an important role in atherosclerotic plaque formation, communicate to each other via exosomes. Endothelial cells released exosomes enriched with mi-143/145 were transferred to smooth muscle cells and therefore in a further experiment injected of miR-143/145 containing exosomes in a mouse model of atherosclerosis resulted in a reduction of atherosclerotic lesion formation[199].

MicroRNA & IR injury

MicroRNAs are small (~22 nucleotide RNA sequences) non-coding RNA that regulates gene or protein expression, usually by inhibition, through complementary base-pair binding with mRNA. There are over a 1000 estimated miRNA genes with over 700 discovered to this date[233]. MiRNA have been implicated in both ischaemia reperfusion injury and heart failure[234]. The ability to regulate specific miRNA and their function could present a novel cardio-protective strategy. Several miRNA have been shown to be up or down regulated following IR injury thus may play an important mechanistic role and target for cardio-protection (Table 1 – adapted from Weiss et al[234]). The potential for clinical application of microRNA both as use as a treatment strategy as well as novel biomarkers are just now being explored. Exosomes that carry miRNAs in the circulatory system may play a pivotal role.

Table 1 - Table of microRNA associated with ischaemia reperfusion injury adapted from Weiss et al.

Down Regulated	Up Regulated
miR – 21[235]	miR-21[235,236]
miR – 1[237]	miR-1[238,239]
miR- 494[240]	miR-494[240]
miR- 133[237]	miR-24[241]
miR – 320[242]	
miR-199a[243]	

CHAPTER 2

Methods and Materials

Ethics Approval

Ethics approval for recruitment of human participants and use of human plasma for exosome experimentation was obtained through successful application to the Research and Ethics Committee (REC reference: 13/LO/0222) and permission for the project was obtained from the University College London Hospital NHS/HSC Research and Development department**.** Please see appendix for full ethics proposal.

Human Participants

All participants were recruited after poster advertisement of the project. Each was given a research and ethics committee (REC) approved participant information sheet detailing the objectives and rational of the project. All participants were given consent REC approved consent forms for signature before recruitment into the study. Inclusion criteria and exclusion criteria for recruited participants were as follows:

Table 2 - Inclusion and exclusion criteria for human participant recruitment.

Inclusion Criteria	Exclusion Criteria
Male, Age <35	Female or Age <18,
Healthy & free of chronic disease	Chronic disease or illness
Not on any medication	On regular medication

Exosome Isolation

The optimal method of exosome isolation, purification and characterization remains uncertain and subject to continuing investigation within the exosome scientific community. There are currently four main approaches to exosome isolation and purification:-

1. Immune-affinity capture
2. Size filtration: (series of filters down to 100 nm pore size followed by centrifugation to concentrate)
3. Size exclusion
4. Ultracentrifugation

Each has its advantages and disadvantages. Immune–affinity capture has the advantage of high specificity yet this comes at as a cost of poor yield. While isolation based of size filtration has the advantage of high yield though this often compromises quality of sample. Over the years the most generally accepted method for exosome isolation appears a process of serial centrifugation followed by ultracentrifugation and subsequent density purification (2). At the start of this project few papers had shown a consistent method for exosome isolation from particularly from human plasma (1). Interestingly in the paper by Caby et al participants donated over half a liter of blood for isolation and characterization of exosomes. The challenge for this project lay with isolating samples with good purity and yield from significantly smaller blood volumes.

Blood Sample Collection

Early morning samples were collected from participants who had fasted from the night before and had not performed any strenuous activity that morning. Blood was taken from the antecubital vein using BD Vacutainer® stretch latex free tourniquet and a BD Vacutainer®Safety-LokTMTMblood collection sets with pre-attached holders. Blood was collected directly into a 4.5ml BD Vacutainer® glass plasma tube, blue conventional closure (Additive: Citrate solution, 0.5ml; Sodium citrate, 12.35mg; Citric acid 2.21mg (Equiv. to 3.2% Sodium citrate)). The blue citrate blood collecting tube was used to prevent platelet activation.

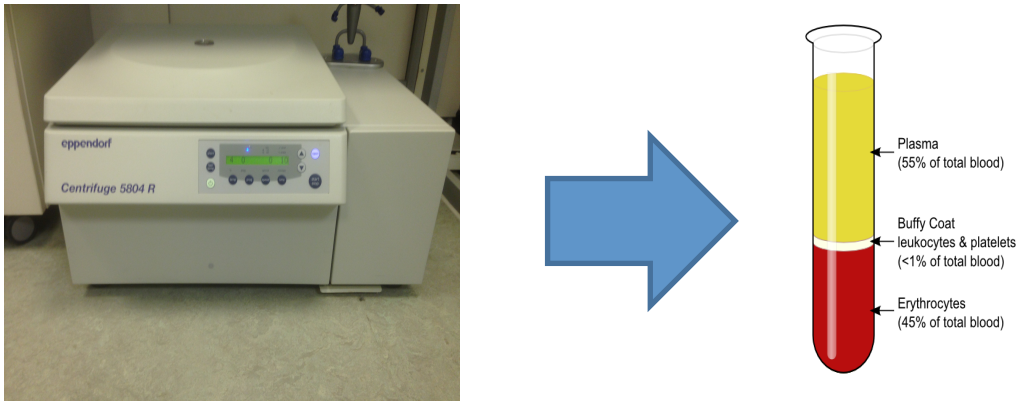
Figure 6 | **BD Vacutainer®Safety-LokTMTMblood collection sets with pre-attached holders.** Blood was collected directly into 4.5mL BD Vacutainer® glass plasma tube, blue conventional closure.

Differential Centrifugation

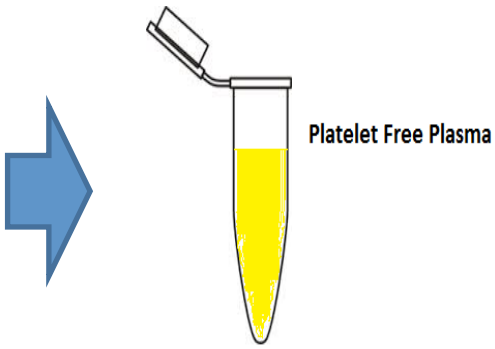
Differential centrifugation allows the isolation of populations cells, organelles or vesicles through the ability to separate on the basis of mass.

To isolate exosomes the blood samples underwent a process of differential centrifugation as follows:

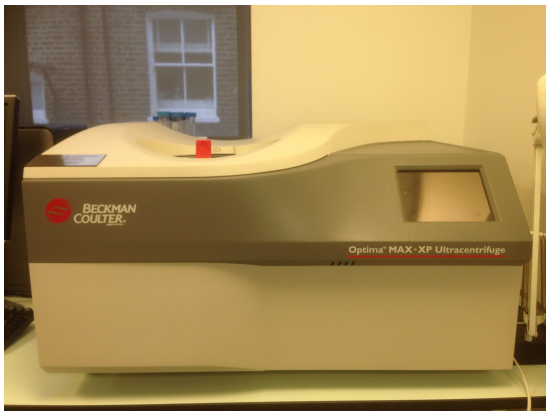
1. Blood was centrifuged at 1,600 x g for 20 min at room temperature to obtain plasma. (Platelet activation is less at room temperature than at 4 ^{o}C).

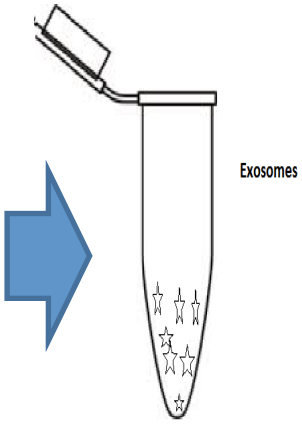


2. Plasma was then removed and centrifuged at 10,000 x g for 30 min at 4 °C to remove cells and platelets.



3. Platelet-free plasma was then transferred to ultracentrifuge tubes for three wash steps, first at 100,000 x g for 90 min, 4 °C with a SW-41 rotor and then for two further times at 100,000 x g for 60 min at 4 °C. After each ultracentrifuge step, supernatant was discarded and pellet re-suspended and washed with PBS.





Remote ischaemic preconditioning protocol

The established RIPC protocol of 3 cycles of 5 minutes arm cuff inflation followed by 5 minutes deflation (reperfusion) was used on participants to obtain RIPC exosomes. This procedure is well tolerated. Blood was collected from one arm at baseline, then the second arm subject to RIPC, and the second blood sample isolated from this arm.

Exosome Characterisation

Electron Microscopy

Electron Microscopy (EM) was performed in collaboration with Dr Mark Turmaine (UCL) for preparation of the electron micrographs. Electron microscopy remains the only technique of direct visualization of exosomes. The technique involves the use of an electron beam to illuminate the exosomes and produce a magnified image. When compared to light microscope, EM has a much greater resolving power and can visualize much smaller structures as electrons have wavelengths about 100,000 times shorter than visible light photons. Exosomes were isolated as per the protocol above however for clearest pictures exosomes were re-suspended in sterile water rather than PBS (which tended to crystalize when dried). Electron microscopy was carried out on a Joel 1010 transition electron microscope (Joel Ltd, Warwickshire, UK) after a standard staining procedure with 0.5% uranyl acetate.

Protein Assay

To ascertain the protein yields from exosome isolations the protein content was determined using bicinchoninic acid (BCA) protein assay (Sigma, UK) and an automated 96-well plate reader (Fluostar Omega, BMG Labtech). The assay works on the premise of formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. The amount of this reduction of the Cu^{2+} to Cu^{1+} is proportional to the protein present. BCA forms a purple-blue complex with Cu^{1+} in alkaline environments and thus the intensity of the purple-colour is proportional to the level of protein in the sample. Exosome samples and samples of bovine serum albumin with established protein concentrations (0-2mg/ml protein) were loaded on to a 96 well plate. BCA and $CuSO_4$ solution was then added and the plate incubated for 30 min at 37 ºC with agitation. FLUOstar Omega microplate reader (BMG Labtech, USA) was then used to measure the colour intensity and the exosome sample protein contents were quantified by comparison to the normalised curve of BCA colour of the known concentrations of BSA samples. All samples were run in duplicate.

Western Blot Analysis

Western blot analysis is the most widely used scientific technique of protein analysis of a tissue homogenate or extract. The process involves gel electrophoresis to separate

proteins according to molecular weight. The proteins are then transferred to a membrane and detection of proteins using targeted antibodies.

Gel Electrophoresis

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using the Mini Protean III system (BioRad, UK) to separate proteins. SDS in the sample buffer denatures multimeric proteins and polypeptide chains so that all proteins have similar charge:mass ratios. This allows the migration of proteins to be determined by size. The addition of an electrical field causes migration of the smaller proteins faster down the gel, thus proteins are separated according to molecular weight. The polyacrylamide gel used for protein separation was prepared by the polymerization of acrylamide and bis-acrylamide. Ammonium per sulfate (APS) and tetramethylethylenediamine (TEMED) was added to initiate polymerization. 4 ml of resolving gel was poured between two glass plates held in a casting frame. To ensure a flat surface 50% isopropanol was pipetted on top of the unset gel. The gel was left for 10 min to set. Once set the isopropanol was gently poured off. TEMED and APS was added to the stacking gel and the stacking gel was then quickly pipetted on to the top of the resolving gel with the insertion of a 12 well comb into the stacking gel to provide the sample loading wells. The gels were then left to set for 5 min. Once set, the gels were placed in a running tank (BioRad, UK) and submerged in running buffer (0.25mM Tris-Base, 0.19M glycine, 0.1% SDS) and the combs removed. In the far left well 8µl of molecular weight reference marker (BioRad, UK) was then loaded to allow determination of protein sizes. Exosome samples were diluted 1:1 with Laemlli buffer (BioRad, UK) and heated to 90°C. 20 µl samples were then loaded in to sample wells. Gels were run at 180 volts for 1 hour or until separation of the protein ladder and the bromophenol blue dye had run off the gel.

Protein Transfer to Membrane

Hybond electrochemiluminescence (ECL) (GE Health Care, UK) western blot transfer paper was used for the transfer of proteins from the acrylamide gel. Nitrocellulose transfer paper and Whatman blotting paper were activated via pre-soaking in 10ml 100% methanol then 1x transfer buffer:

- 10% 10x transfer buffer (10x transfer buffer = Glycine 144.2g, Tris 30.3g, in 1litre distilled water)
- 20% MeOH and
- 70% water.

The gels were removed from the glass plates and the stacking gels carefully discarded. The gel was then carefully placed on to the activated transfer paper and sandwiched in between Whatman blotting paper and loaded on to the transfer tank. The sandwich of gel, transfer and blotting paper were further soaked with 1% transfer buffer with air bubbles gently removed by rolling a 5ml pipette across the top surface. The gel was then set to transfer over 45min at 10V with a current of 1A.

Membrane Blocking and Protein detection

To reduce non-specific binding once protein transfer has taken place, the nitrocellulose membrane was blocked for 1 hour with 5% bovine serum albumin (BSA) in PBS–Tween (PBS Tablets (Sigma, UK) containing: 10mM phosphate buffer, 2.7mM KCl, 137mM NaCl and 0.1% Tween 20 (Sigma, UK)). The membrane was then washed PBS-Tween for 45 minutes. The PBS-Tween was changed every 15 minutes. The membrane was then incubated overnight with a horseradish peroxidase (HRP)-primary antibody of choice diluted to manufacturer's guidelines. The next morning the membrane was washed again with PBS-Tween for 45 minutes as per previously described. The (HRP) –secondary antibody was then added to the membrane and incubated for 1 hour. The membrane was then washed with PBS tween for 45 minutes as per previously described. The membrane was then finally incubated in ECL reagent (GE healthcare UK) as per manufacturers protocol, to start the chemiluminescences reaction. The incubation of the HRP linked secondary antibody and the ECL agent causes the light emitting oxidation of luminol. This light emission was used to detect and localize the primary antibody that should be bound specifically to the protein of choice on the membrane. After ECL incubation the membrane was sandwiched in between Saran wrap and placed in an X-ray film cassette (Kodak, UK) and exposed to Hyperfilm ECL high performance chemiluminescence film (GE Healthcare, UK). Exposure time was tailored to achieve optimal band intensity and the ECL film is then placed in developer followed by fixer solution (Sigma UK). A Black band on the ECL film indicates the presence of protein of interest. Films were left to dry and scanned on to the computer.

Fluorescence-activated cell sorting (FACS) analysis

Flow cytometry is a laser-based technique that allows for the counting and sorting of cells or particles for experimental purposes, as well as for biomarker detection. Particles are suspended in a stream of fluid and passed through a laser. The technology allows for the simultaneous measurement of multiple light-based physical

characteristics of cells or particles, including intrinsic light emission as well as stimulated fluorescence emission. The flow cytometer is composed of three main components:

- **Fluidics:** that which transports particles/cells through the laser.
- **Optics:** a laser, which illuminates the cells and fluorescence detectors.
- **Electronics:** which converts the detected light signals into data that can be processed by the computer and its dedicated software.

Flow cytometry then, as a brief summary, allows the analysis of cells or particles in a fluid. However, there is a size limitation of this technique, which can only detect particles in the range of 0.2 μm to 50 μm. The size of exosomes of 0.05-0.1 μm does not allow direct analysis through FACS. To overcome this problem, exosomes were conjugated to microspheres (4μm) (Aldehyde/Sulfate Latex Beads, 4% w/v, 4 um Molecular Probes), a size that is in the detection range of a flow cytometer. The method used to analyze exosomes involved the following five steps:

1. Nanosight analysis for quantification of exosomes (methods described later)

   Accounting for the size of the microsphere beads, the number of exosomes (50-100 nm in size) needed to fully coat the beads was estimated to be ~$2x10^9$ exosomes. Therefore, after Nanosight quantification of each exosome sample, the volume of sample needed to coat the microspheres with at least $2x10^9$ exosomes was calculated.

2. Clearing of macromolecular IgG complexes

   The next step was the clearing of macromolecular IgG complexes present in the plasma, which are present also in the exosomal samples after purification by ultracentrifugation, and they can bind to the microspheres creating the risk of unspecific artifacts in the FACS analysis. To overcome this, the exosome sample was first cleared of IgGs using G protein sepharose beads. The volume calculated from nanosight analysis was then added to PBS solution and 20 μl of G protein sepharose beads to make up a 1ml sample. This sample was then rotated at 4°C for 60 min and centrifuged 9000 rpm, 4 min, 4°C. The supernatant containing cleared exosomes was used for binding to the aldehyde microspheres.

3. Binding of exosomes to microsphere beads.

   The next step is the binding and conjugation of exosomes to microspheres beads, whose size ranges inside the detection limits of the FACS. Following the previous centrifugation, the supernatant (cleared exosomes) was taken into a new tube and 5 µl of aldehyde beads (Aldehyde/Sulfate Latex Beads, 4% w/v, 4um Molecular ProbesR)) were added. This was then incubated for 1 h at 4°C with rotation for binding of the exosomes to the microspheres. Once incubated the sample was then centrifuged at 9000 rpm, 4 min, 4°C. The supernatant was removed and 1ml PBS added. Centrifugation and removal of supernatant was repeated a further two times as washing steps.

4. Binding to Primary and Secondary antibodies

   The exosome-coated microspheres were re-suspended and blocked in 1 ml of 1% BSA/PBS 0.1% NaN3 (blocking buffer). This was then left to rotate at 4°C for 1 hour. 100 µl aliquots of the blocked beads were then transferred into new tubes. These aliquots were then incubated with different primary antibodies for 1 h at 4°C for the detection of specific markers. The microsphere beads that had been stained with primary antibody were washed three times through a process of centrifugation at 9000 rpm, 4 min, 4°C in blocking buffer. After the third wash the samples were re-suspend in 100 µl blocking buffer and incubated with the appropriate fluorescent secondary antibodies for 40 min at 4°C (Anti-Rabbit Alexa 488/ Anti-Mouse Alexa 488). Once incubated with the secondary antibody, the exosome-coated beads were washed 3 times by centrifugation and finally re-suspended in 100 µl blocking buffer, working in a dark environment to prevent fluorescence photo-bleaching. The optimal dilutions for all antibodies were titrated, after a series of experiments with different titers of primary and secondary antibodies.

5. Analysis on the Flow Cytometer

   The microsphere beads were then analyzed on a flow cytometer (Accuri C6, BD Biosciences) using the software Cflview PlusAnalysis (BD Biosciences). Each aliquot was aspirated into the flow cytometer and results were presented graphically. From the initial forward scatter *vs.* side scatter plot (FSC/SSC), the population of beads was gated. A second graph of pulse width vs. SSC plot allowed the gating of singlet's of beads. A third plot showed the specific fluorescence of each set of beads, according to the different antibodies used. Results were

presented as histogram plots, against control experiments using beads that were blocked with BSA, in the absence of exosomes. On each experiment, control samples with no exosomes were used.

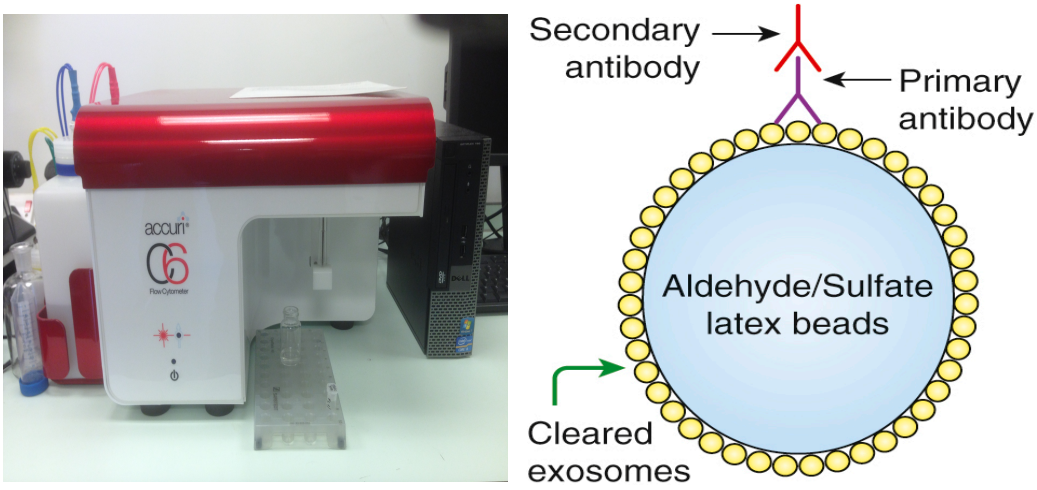
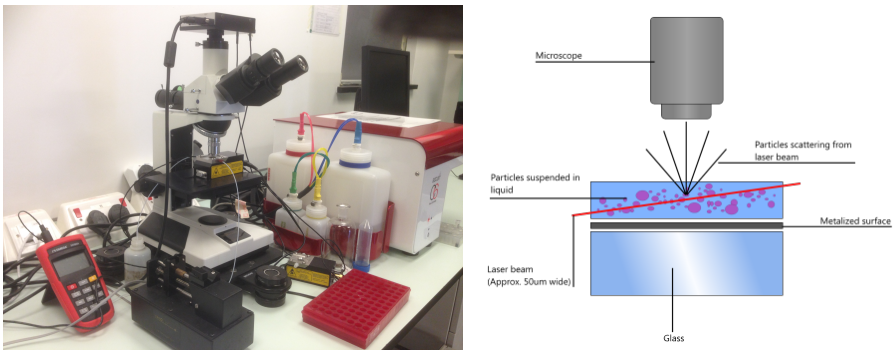


Figure 7 | **A) Accuri C6, BD Biosciences flow cytometer. B) Diagram depicting Aldehyde/Sulfate Latex Beads with bound exosomes and primary and secondary antibody.**

Nanoparticle Tracking Analysis

For many years a reliable methodology of exosome quantification had not been developed and accepted. At the start of this project a new apparatus called the "Nanosight" a nanoparticle tracking analysis (NTA) machine had entered the market. Its manufacturers claimed the ability to accurately visualize and quantify particles in liquids from 10-2000nm. NTA analysis is based on the principle that the rate of Brownian movement of nanoparticles in a solution is related to their size. When illuminated by a 405nm laser the scattering of light by the particles is measured using a microspore and scientific digital camera. Software produced specifically tracks each particle and using the Stokes-Einstein equation the rate of particle movement is related to a size/sphere diameter of the particle. The technique thus allows for the calculation of number of particles in a given solution and the distribution of particles in relation to size of particles. Exosome samples were diluted in 1ml PBS to achieve optimal concentration for nanosight analysis as per manufacturers guidelines based on visual

interpretation of the number of particles on viewing screen. If the sample was too concentrated the nanosight's ability produce accurate results was affected. From this, the particle concentration of the original sample was calculated. The diluted sample was then injected into sample chamber of the Nanosight LM10 unit (Nanosight Ltd, Amesbury, UK) while holding the chamber vertically ensuring that no air bubbles were present. The 430nm laser was then switched on and visual parameters were optimized as per manufacturer's guidelines. The rest of the sample (in 1ml syringe) was placed on the electronic syringe set to start continuous slow injection of sample through the chamber. Three 30-second videos were recorded for each sample and analyzed by the Nanoparticle Tracking Analysis (NTA) 2.2 software. After each sample analysis the chamber was dismantled was cleaned thoroughly with 70% ethanol and dried before the insertion of the next sample.



A **B**

Figure 8 **A) Photograph of nanosight LM10 unit. B) Nanosight microscope unit uses a laser light source to illuminate particles within a 0.3 ml sample, introduced to the viewing unit with a disposable syringe. Particles appear individually as point-scatterers moving under Brownian motion, which are then visualized on a computer screen and quantified using the nanosight software provided.** Diagram taken from www.Nanosight.com

MicroRNA isolation and Analysis (collaboration with Centre of Cardiovascular Genetics, UCL. Professor Steve Humphries and Anastasia Z. Kalea)

The immediate research group within the Hatter institute did not have the expertise in miRNA isolation and analysis thus collaborations were formed with the Centre of Cardiovascular Genetics at UCL working with Professor Steve Humphries and

Anastasia Z. Kalea. Human plasma exosomes were isolated as per isolation protocol described previously in this thesis at the Hatter Institute. After various optimization experiments human plasma exosomes, isolated by ultracentrifugation, were finally re-suspended directly in miRVana homogenization buffer to improve quality and yield of RNA. miRNA isolation was carried out at the Centre of Cardiovascular Genetics using the miRVana kit (Ambion, Austin, TX, USA). The RNA was further purified with 4 ethanol washes and RNA pellet re-suspended in 20 µl of distilled H_2O. Finally RNA was purified further with DNAse I treatment. miR expression measurements were carried out using real time-PCR.

Ischaemia Reperfusion Invitro Methods

Primary cardiomyocyte isolation (Carried out by Jose Vincencio, Hatter Cardiovascular Institute)

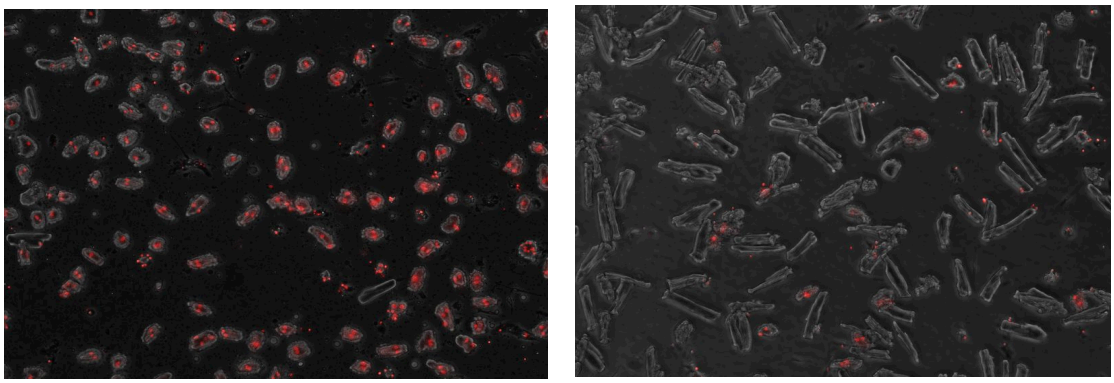
Male Sprague Dawley rats (aged circa 4 months) were used in these studies and were obtained from Charles River UK. Animals were treated in accordance with the Animals (Scientific Procedures) act 1986 published by the UK Home Office and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996). Rats were anesthetized with 300 µl of intra peritoneal sodium pentobarbital. The hearts of adult rats were isolated, placed in an ice-cold buffer and perfused via the aorta on a non-recirculating perfusion apparatus. Perfusate used was a modified calcium-free Krebs-Ringer-HEPES (KRH) buffer (in mM): 116.0 NaCl, 5.4 KCl, 0.4 MgSO4, 20.0 HEPES, 0.9 Na2HPO4, and 5.6 glucose (pH7.4). The hearts were perfused at 14ml/min for 5 minutes with KRH buffer containing 1 mg/ml BSA and 3.3 µM EGTA. The perfusate was bubbled with 100% oxygen with the temperature maintained at 37°C. The isolated rat hearts were then perfused for 15minutes with Worthingtontpe II collagenase and 25 µM calcium. Lastly the isolated hearts were perfused with KRH buffer containing 50µM calcium for 5 min. The hearts were then dismounted from the perfusion apparatus. The atria were first trimmed away leaving the ventricles to be then minced. The ventricles then underwent a series of further digestions with collagenase and then filtered through a nylon mesh. Finally the cells were washed with restoration buffer: KRH buffer plus 10 mg/ml BSA, 0.5 mM Na-pyruvate, 5.0 mM taurine, 2.0 mM carnitine, 1.0 mM creatine, and 75 µM calcium. Cells were then placed onto laminin coated round coverslips and incubated overnight at 37°C in an atmosphere of 95% air-5% CO2 in M-199 medium (M7653, Sigma) containing 10% fetal calf serum and 1% penicillin-streptomycin (Sigma). The following day, cells were washed and kept in restoration buffer.

In vitro Ischaemia Reperfusion Testing *(Carried out in conjunction with Jose Vincencio and Jessica Kearney, Hatter Cardiovascular Institute)*

Ischemia and reperfusion was simulated by firstly placing the cardiomyocyte cells in an ischaemic buffer containing 128 mmol/L NaCl, 2.2 mmol/L $NaHCO_3$, 14.8 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L K_2HPO_4, 1 mmol/L $CaCl_2$, 10 mmol/L Na. lactate (pH 6.4). The cardiomyocyte cells were then placed in a hypoxic chamber in which the air was replaced 95% N_2/ 5% CO_2 for 3 h. Post 3 hours the cardiomyocyte cells were removed and placed into a standard incubator in normal medium. For controls, cardiomyocyte cells were incubated in normoxic medium containing 118 mmol/L NaCl, 22 mmol/L $NaHCO_3$, 2.6 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L K_2HPO_4, 1 mmol/L $CaCl_2$, 10mmol/L glucose (pH 7.4 gassed with 95% O_2 / 5% CO_2). The five different groups tested were:

1. Control cardiomyocytes placed in normoxic buffer
2. Cardiomyocytes + Ischaemia Reperfusion
3. Cardiomyocytes + Ischaemia Reperfusion + Control Human Plasma Exosomes (concentrations of 0.5µl to 0.0005µl)
4. Cardiomyocytes + Ischaemia Reperfusion + RIPC Human Plasma Exosomes (concentrations of 0.5µl to 0.0005µl)
5. Cardiomyocytes + Ischaemia Reperfusion + Insulin

Cardiomyocytes cells were then analyzed under the microscope and the percentage of cell death was calculated.



A **B**

Figure 9 | **Image of A) largely dead myocytes (red dots) and B) Healthy (alive) myocytes.**

CHAPTER 3

Results

Isolation technique and characterisation of Exosomes

Introduction

The optimal method of exosome isolation, purification and characterization remains uncertain and subject to continuing investigation. Exosome isolation using commercially available polymeric precipitation kit, Exoquick was briefly assessed. However, based on the most widely accepted, current recommendations differential centrifugation was chosen as the method for exosome isolation from human plasma and evaluated in the following experiments[244].

Aims

The aim for these experiments was:

1. To characterize and confirm the presence of exosomes isolated by the method of differential centrifugation using established methods:

    a. Electron microscopy
    b. Protein and Western Blot Analysis
    c. Nanoparticle tracking analysis (to establish validity and use as a tool for quantification of exosomes)

d. Flow cytometry

2. To optimise the isolation technique for exosomes from human plasma and determine optimum storage conditions for future experiments.

3. Establish the presence of, and isolate microRNA from human plasma exosomes.

Results

To characterize and confirm the presence of exosomes isolated by the method of differential centrifugation using established methods:-

Electron Microscopy

Detailed Methods

Early morning blood samples were collected from participants who had fasted from the night before and had not performed any strenuous activity that morning. Blood was taken from the antecubital vein using BD Vacutainer® stretch latex free tourniquet and a BD Vacutainer®Safety-LokTMTMblood collection sets with pre-attached holders. A total of 72ml Blood was collected directly into a 4.5ml BD Vacutainer® glass plasma tube, blue conventional closure (Additive: Citrate solution, 0.5ml; Sodium citrate, 12.35mg; Citric acid 2.21mg (Equiv. to 3.2% Sodium citrate)). 16 x 4.5mL BD Vacutainer® glass plasma tubes containing blood was then centrifuged at 1,600 x g for 20 min at room temperature to obtain plasma. The plasma was then removed and placed in 2ml eppendorf tubes and centrifuged at 10,000 x g for 30 min at 4 °C to remove cells and platelets. Platelet-free plasma then transferred to ultracentrifuge tubes for three wash steps, first at 100,000 x g for 90 min, 4 °C with a SW-41 rotor and then for two further times at 100,000 x g for 60 min at 4 °C. After each ultracentrifuge step, supernatant was discarded and pellet re-suspended and washed with water. Electron microscopy was carried out on a Joel 1010 transition electron microscope (Joel Ltd, Warwickshire, UK) after a standard staining procedure with 0.5% uranyl acetate. Electron Microscopy (EM) was performed in collaboration with Dr Mark Turmaine (UCL) for preparation of the electron micrographs.

Result

By electron micrography of the exosome preparations, we observed the classical concave “cup shaped vesicles” within the expected size range (50-100nm) of exosomes (Fig 10).

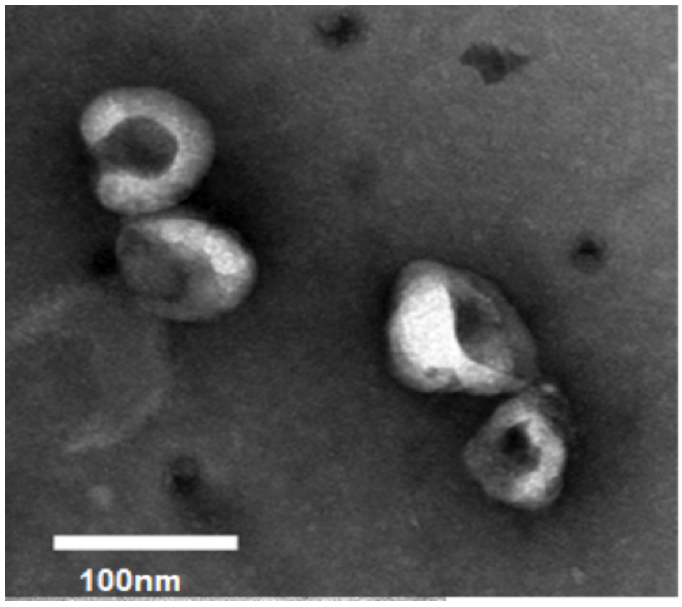


Figure 10 **Successful isolation of exosomes from human plasma through differential centrifugation as evidenced by electron microscopy. Electron micrographs of purified human plasma exosomes.** Bar 100 nm.

Discussion

The presence of cup shaped vesicles by electron microscopy (Fig 10), of the expected size range (50-100nm diameter), confirms successful isolation of exosomes from human plasma through the protocol of differential centrifugation. Although the vesicles are believed to be spherical in solution, they have the appearance of collapsed vesicles after drying.

Western Blot Analysis of exosomal proteins

As per convention we also sought to confirm successful exosome isolation by the presence of known exosomal protein markers using western blotting. For future experiments it was also important to ascertain a protocol for exosome isolation that would produce a relatively pure sample, with the trade-off that the yield remain

reasonably high. The amount of exosomal protein in human plasma is likely to be dwarfed by the abundance of other serum proteins. Albumin is the most abundant blood protein, and thus one of the potential contaminants. Using human albumin as a marker of serum protein and a surrogate marker for impurity, serial ultracentrifuge washing steps were carried out to identify the number of washes needed to obtain the cleanest sample while still acquiring adequate protein concentrations for further experimentation.

Specific Aims

1. To ascertain the number of ultracentrifuge washes that produces the purest sample of exosome while retaining enough protein for analysis.
2. To confirm that the isolation technique produce exosomes as evidenced by known markers of exosomes on western blot analysis

Ascertain the number of ultracentrifuge washes that produce the purest sample of exosome while retaining enough protein for analysis.

Detailed Methods

Exosome isolation

Exosomes were isolated from 54ml of human blood (12 x 4.5mL BD Vacutainer glass plasma tubes) as described above (electron microscopy experiment) up until the ultracentrifugation step. After centrifugation to produce platelet free plasma, this is then transferred to 6 ultracentrifuge tubes (4ml plasma per tube) and made up to 8ml with sterile PBS for wash steps at 100,000 x g for 90 min, 4 °C with a SW-41 rotor. After the first wash step one tube removed, supernatant discarded and pellet re-suspended in 1ml PBS. Sample labelled as Sample 1. The supernatants of the five remaining tubes were discarded and the pellet re-suspended in 8ml PBS and put forward for a further ultracentrifuge wash. This process was repeated for further 4 ultracentrifuge washes at 100,000 x g for 60 min at 4 °C. After each ultracentrifuge step one tube removed, supernatant discarded and pellet re-suspended in 200µl PBS. Resulting in 5 samples ranging from 1 ultracentrifuge wash to 5. (1 extra sample was kept through the process in case of any errors/spillages)

Protein Assay protocol

Exosome samples were aliquoted into a 96 well plate, and compared with a serial dilution of standard protein concentrations (0-2mg/ml protein) obtained using bovine serum albumin. Bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific) and $CuSO_4$ solution was then added and the plate incubated for 30 min at 37 ºC with agitation. This assay is based on the principle that protein will chelate copper ions (biuret reaction), reducing Cu^{2+} to Cu^{1+}, which reacts with bicinchoninic acid forming an intense purple-coloured reaction product. A FLUOstar Omega microplate reader (BMG Labtech, USA) was then used to measure the light absorbance at wavelength 562 nm. Exosome sample protein contents were quantified by comparison to the normalised curve of BCA colour of the known concentrations of BSA samples. All samples were run in duplicate.

SDS-PAGE Sodium-dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) Western blot experiment protocol

Exosome sample preparation

For Sample 1, 20 µl Exosome sample taken and added to 180 µl of Laemlli buffer (Sigma, bromophenol blue 0.004%, DTT 400 mM, glycerol 20%, SDS 4%, TRIS, 0.125 M), in a 1.5 ml Eppendorff. Samples 2-5, 100 µl Exosome samples taken and added to 100µl of Laemlli buffer in a 1.5 ml Eppendorf tube. All were then boiled at 95 ^{o}C for 10 min to fully denature the proteins, and finally centrifuged at 12,000 rpm for 5mins. Samples were then ready for loading on to the gels.

Gel Preparation

10% polyacrylamide resolving gels were made by mixing 3.75 ml of 1 M Tris-HCl pH 8.8 with 3.34 ml 30% Acrylamide, 2.8 ml distilled H2O and finally 100 µl of 10% SDS in a 50 ml Falcon tube. 7.5 µl of TEMED and 75 µl APS mixed together then added to the gel mixture just prior to pouring between glass gel plates to give the gels a thickness of 0.75 mm. Approximately 4 ml of resolving gel was added between each set of plates, giving a gap of ~0.5 cm between the top of the resolving gel and the bottom of the wells in the stacking gel. Butanol was gently pipetted on to the unset resolving gel to ensure it set flat. After the resolving gel had set, the butanol was removed Stacking gel made up by mixing 0.625 ml 1 M Tris-HCl pH 6.8 with 0.75 ml 30% Acrylamide, 3.5 ml Water and 50µl 10% SDS in a 10 ml Falcon tube. Similarly 7.5µl TEMED and 75µl APS were added to the stacking gel, before it too was poured between the plates. 12-well combs were placed into the stacking gel to ensuring loading of samples could occur.

Gels were immersed in running buffer (0.25mM Tris-Base, 0.19 M glycine, 0.1 % SDS). 20 µl Exosome samples and 7 µl of molecular weight markers (BioRad, UK) were loaded on to the stacking gel. Applying 180 V separated proteins electrophoretically across the gel until the bromophenol blue dye had run through the gel (~ 1 h).

Protein transfer

Proteins were then transferred onto PVDF membranes (Amersham, UK) pre-wetted in methanol and equilibrated in transfer buffer (25 mM Tris-base, 0.19 M gylcine, 20 % v-v methanol, 0.1 % w-v SDS). Resolving gels were placed on top of PVDF membranes and sandwiched between 2 pieces of blotting paper (also pre-soaked in transfer buffer) and placed in a semidry transfer cell (BioRad, UK). Air bubbles were removed by rolling a disposable serological pipette across the preparation before transferring at 10 V for 45 min with the current limited at 250 mA per gel.

Zinc Stain

PVDF membrane added to Pierce Zinc Reversible Stain for 10 min then remove. Zinc developer solution added to membrane for two minutes then removed. 10 ml sterile water added. To erase the stain, Zinc Stain eraser solution was added and left to rock for 5 minutes. Membrane then washed 3 x 5 min with 10 ml sterile water.

Membrane Blocking and Incubation with Human Albumin antibody:

PVDF Membrane blocked with 5 % Bovine Serum Albumin (BSA) PBS solution 1 h. Membrane then incubated in 1:1000 anti-albumin antibody ab19194 (Cell Signalling) with gentle rocking overnight in cool environment. Membrane then washed 3 x 15 min with PBS-0.1% Tween. Rabbit anti-albumin antibody (Cell signaling) was then added and incubated for 1 h with gentle rocking. The secondary antibody was then discarded and the membrane washed again 3 x 15 min with PBS-0.1%Tween.

Western Blot Analysis

The membranes were analyzed on a Li-Cor Odyssey infrared fluorescent imaging system.

Results

We observed a decreasing yield of protein with increasing numbers of ultracentrifugation steps (Table 3, Fig 11).

Table 3 - Table of calculated protein yield (µg) per 1 ml plasma from isolation samples using differing number of ultracentrifugation wash steps.

Number of ultracentrifuge steps	Protein Yield (µg) per 1 ml plasma
1	969.7
2	35.4
3	14.3
4	3.8
5	1.5

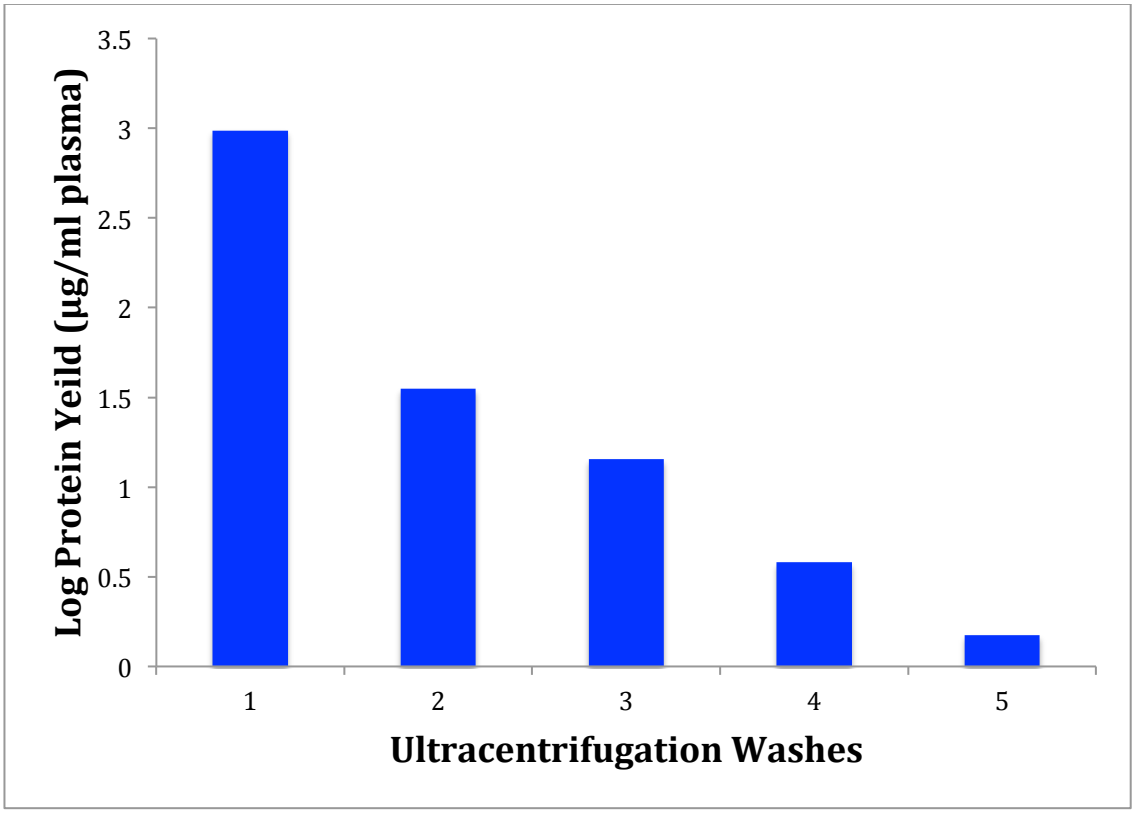


Figure 11 Graphical bar chart representation of calculated protein yield (µg) per 1 ml plasma from isolation samples using differing number of ultracentrifugation wash steps.

To determine albumin content the protein was separated by SDS-PAGE and albumin content measured by Western blot analysis. We observed a decreasing albumin signal with increasing number of ultracentrifugation washes.

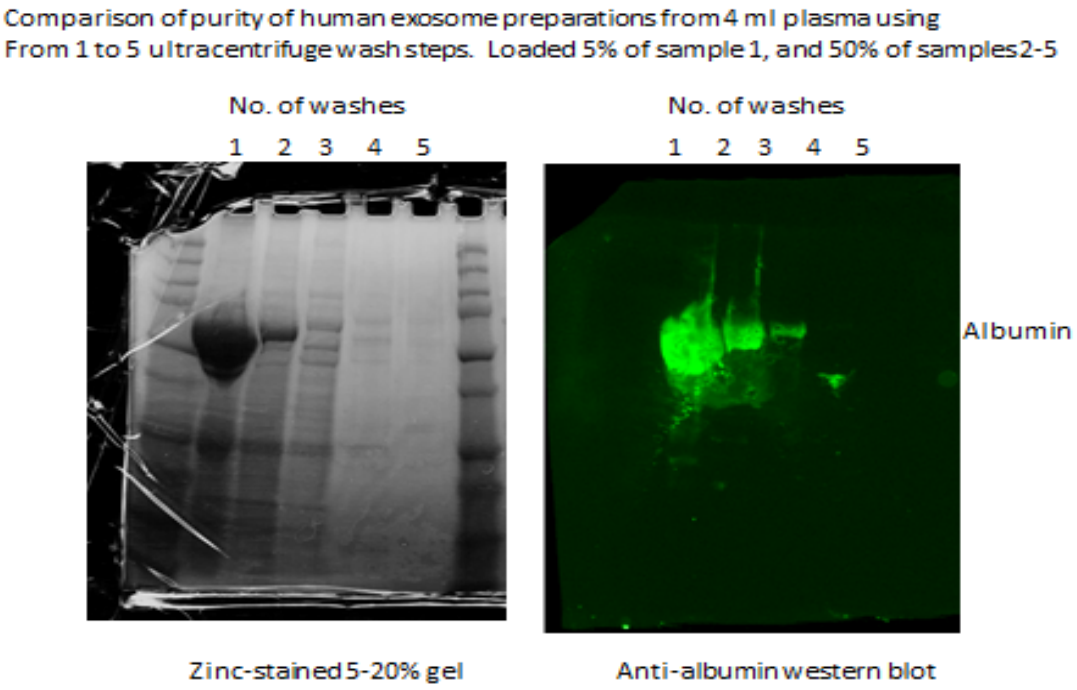


Figure 12 | **Comparison of purity of human exosome preparations from 4 ml plasma using 1-5 ultracentrifuge steps. A) Zinc stain B) Anti-albumin western blot.**

Discussion

From the above experiments we conclude that 3 ultracentrifuge washing steps with PBS produces a sufficient pure exosome sample from human plasma while maintaining enough exosomal concentration for further analysis.

Does isolation technique of exosomes from human plasma produce exosomes as evidenced by known markers of exosomes on western blot analysis?

Detailed methods

Human Exosome Isolation

Exosomes were isolated from 81ml of human blood (18 x 4.5mL BD Vacutainer glass plasma tubes) as described previously up until the ultracentrifugation step. Platelet free plasma was then transferred to 6 ultracentrifuge tubes (6ml plasma per tube) and centrifuged at 100,000 x g for 90 min, 4°C with a SW-41 rotor. The supernatant was discarded leaving ~ 10µl and then re-suspend in 8 ml PBS. A further ultracentrifuge wash step was undertaken at 100,000 g 60 min, 4°C in Beckman Optima L in SW-41

for 1 hr. The supernatant was discarded leaving ~ 10µl and re-suspend in 8 ml PBS. The final ultracentrifuge wash step was undertaken at 100,000 g 60 min, 4°C in Beckman Optima L in SW-41 for 1 hr. Exosome pellets (non–visible) were re-suspended in 500µl sterile from 6 centrifuge tubes and combined to form 1 sample

SDS-PAGE Sodium-dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) Western blot experiment protocol:

Exosome sample preparation

A 100µl Exosome sample was taken and added to 100µl of Laemlli buffer 1.5ml Eppendorff tube and boiled at 95°C for 10 min. The sample was then centrifuged at 12,000 rpm for 5 min and loaded on to gels.

Gel Preparation & Transfer

Gel preparation and protein transfer to membranes was carried out as described previously.

Membrane Blocking and Incubation with Primary and Secondary antibodies

The same experimental protocol was carried out as per previously described. The classical exosomal protein markers HSP70, CD63, CD9 and CD81 were probed using following antibodies and corresponding secondary antibodies:

1. HSP70 (Santa Cruz) 1:200; 1:500 Mouse Secondary Antibody
2. CD63 (Santa Cruz); 1:200; 1:500 Rabbit Secondary Antibody
3. CD9 (Santa Cruz); 1:200; 1:500 Mouse Secondary Antibody
4. CD81 (Santa Cruz); 1:200; 1:500 Rabbit Secondary Antibody

ECL protocol and scientific film development

7 ml GE Healthcare Western Lightning® Chemiluminescence Reagent was added to PVDF Membrane and incubated for 3 min at room temperature with gentle shaking. The Western blot was taken to the developing room and the membrane placed between the covers of a propylene sheet protector with the black interface removed. The lights were switched off and Kodak Scientific Imaging film placed on top of the membrane. The film was exposed for various amounts of time for optimum detection and then developed.

To confirm isolation of exosomes the protein content of isolation samples were separated by SDS-PAGE and classical protein markers for exosomes content measured by Western blot analysis. We were able to detect two of the classical exosomal protein markers HSP70 and CD63, however CD9 and CD81 were consistently undetectable (Table 4, Figure 13).

Table 4 - Table of common exosomal proteins probed in our human plasma exosomes via western blot. HSP70 and CD63 detected though CD9 and CD81 not detected.

Protein	Marker detected?
HSP70	Yes
CD63	Yes
CD9	No
CD81	No

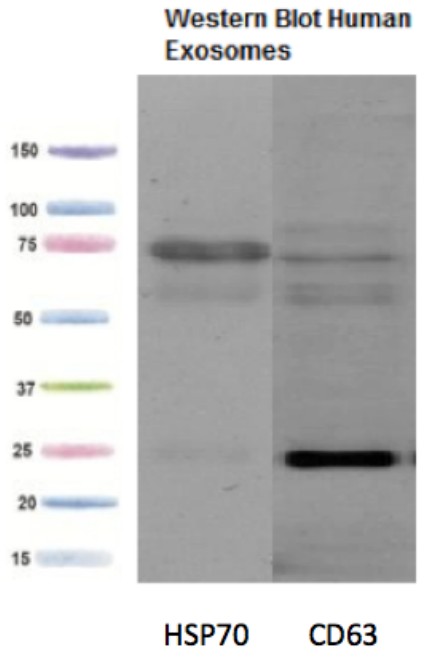


Figure 13 | Western blot of human plasma exosomes showing detection HSP70 and CD63.

Discussion

HSP70 and CD63 were consistently found in exosomes from the plasma from all participants. Interestingly though classically in literature the molecular weight of CD63 in exosomes is described as 53 kDa. We consistently detected a signal at 26 kDa both in human plasma exosomes but also in concurrent work carried out on rat plasma exosomes. On further review of the antibody product data sheet, it describes the molecular weight of CD63 core protein as 26 kDa. The other typical tetraspanins tested, CD9 and CD81 were consistently undetectable in our samples. This again was consistent with the findings of other experiments within our group working on rat plasma exosomes. It is interesting to note the first study by Marie-Pierre Caby et al who demonstrated the presence of these tetraspanins in human plasma through western blotting, isolated exosomes from a far greater sample of blood (600ml as compared to our 81ml)[154], our limiting factor being the size of centrifugation systems. This inability to detect these tetraspanins may be due to insufficient protein concentration or a lack of sensitivity of our primary antibodies. Alternatively, the exact complement of tetraspanin molecules may vary between exosomes of different sources, and CD9 and CD81 may not be present at high concentration in plasma exosomes. We thus conclude through the detection of classical exosomal protein markers HSP70 and CD63 that our isolation protocol successfully isolates exosomes from human plasma.

Exoquick vs Ultracentrifugation method for exosome isolation

A number of easy-to-use and inexpensive commercial kits are also available for exosome isolation such as ExoQuickTM (System Biosystems). The exact methods by which these kits isolate exosomes are not disclosed by their respective companies, but involve selective precipitation by a proprietary polymer. Here we briefly assessed their ability to isolate exosomes and purity.

Detailed methods

Human Exosome Isolation

Ultracentrifugation exosomes were isolated using 3 ultracentrifugation washes while Exoquick exosomes were isolated as per manufactures protocol and guidelines[245]

Results

Both Exoquick and Ultracentrfugation methods successfully isolate human plasma exosomes based on westernblot analysis for known exosome markers HSP70 and CD63. However Exoquick samples appear less pure when probed for human abumin,

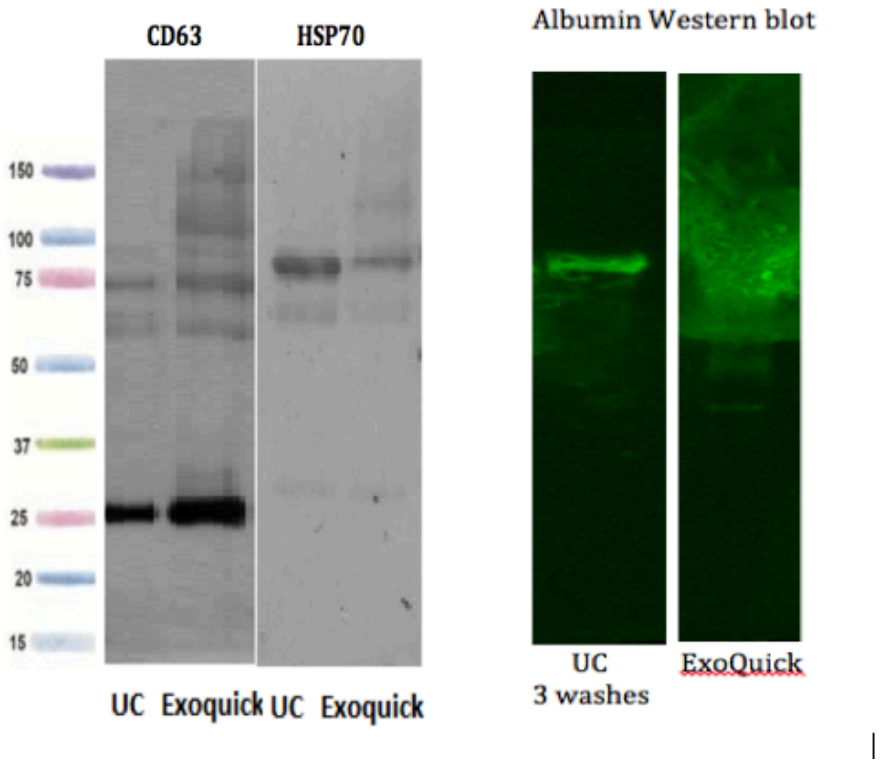


Figure 14 | **Western blot of human plasma exosomes showing detection of HSP70 and CD63 and anti-albumin through ultracentrifugation (UC) and Exoquick (System Biosystems) isolation.**

Discussion

Our pilot study shows that both Exoquick and Ultracentrifugation methods successfully isolate human plasma exosomes based on western blot analysis for known exosome markers HSP70 and CD63. However the purity appears to be low. This concurs with increasing reservation of this isolation technique within the scientific community [246] .

Nanosight Tracking Analysis

As described previously the Nanosight tracking analysis (NTA) equipment is currently the only established method of visualising and quantifying exosomes in a polydisperse sample. In the following experiments the NTA equipment was used to confirm the successful isolation plasma exosomes. Secondly, using the NTA equipment we aimed to establish the variability in exosome numbers produced from our isolation technique. The variability in exosome numbers produced from the protocol of analysing by NTA was also calculated. This provided confidence that we had established a reliable and reproducible method of exosome isolation from human plasma. Lastly while working with limited resources of human samples as well as the time consuming protocol for isolation, it was important to establish the optimal storage conditions for exosomes that would allow for future experiments.

Specific aims

1. Does isolation protocol produce exosomes as per detected by NTA?
2. Using the NTA analysis machine, calculate the variability in numbers of exosomes isolated from our isolation protocol from human plasma. In addition calculate the internal variability of the NTA machine analysis protocol.
3. Using the Nanosight NTA, what is the optimal storage condition of exosomes for future analysis and experiment use?

Does isolation protocol produce exosomes as per detected by the Nanosight?

Detailed Methods

Human Exosome Isolation

Three participants were recruited to participate in this experiment. Human plasma exosomes were isolated using the same protocol used in the previous experiment. Resulting in 3 exosome samples from 3 participants: Labelled 1 to 3.

Analysis on Nanosight

10µl of exosome samples were diluted in 1ml PBS to achieve optimal concentration for nanosight analysis as per manufacturers guidelines based on visual interpretation of the number of particles on viewing screen. The diluted exosome sample was then injected into sample chamber of the Nanosight LM10 unit (Nanosight Ltd, Amesbury,

UK) while holding the chamber vertically ensuring that no air bubbles were present. The 430nm laser was then switched on and visual parameters were optimized as per manufacturer's guidelines. The rest of the sample (in 1ml syringe) was placed on the electronic syringe set to start continuous slow injection of sample through the chamber. Three 30-second videos were recorded for each sample and analysed by the Nanoparticle Tracking Analysis (NTA) 2.2 software. After each sample analysis the chamber was dismantled was cleaned thoroughly with 70% ethanol and dried before the insertion of the next sample. Results were then analysed to calculate number of particles isolated per ml/plasma. Sterile PBS buffer alone was analysed to ensure those particles that were seen were indeed from the exosome isolation sample alone.

Results

I analysed the isolation samples from 3 participants using the Nanosight NTA system. We were able to demonstrate that our isolation sample had staggeringly large particle concentrations with an average of 2.4×10^{11} particles per ml human plasma. The modal sizes of these particles (85-96nm) were also in the exosomal size range (Table 5, Figure 14).

Table 5 - Table of NTA analysis results from the human plasma exosome isolations from 3 participants showing the calculated concentration of particles/ml of plasma and their modal size. Sterile buffer alone shows no particles thus all particles are true reflection of sample selection.

Participant	Concentration (particles/ml plasma)	Modal Size (nm)
Sterile PBS buffer	0	0
1	2.22E+11	96
2	2.13E+11	85
3	2.90E+11	88

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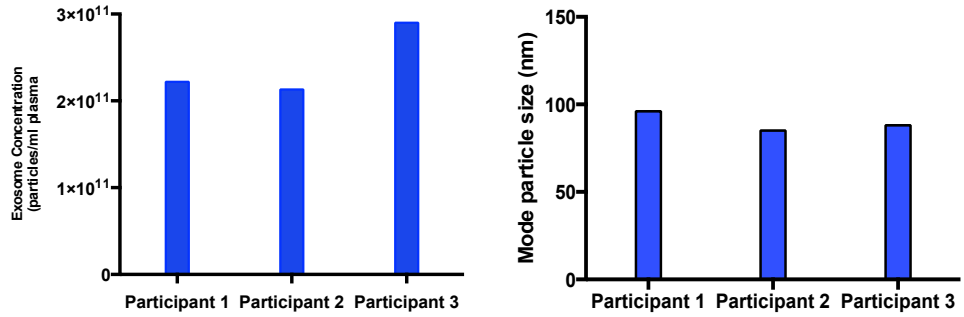

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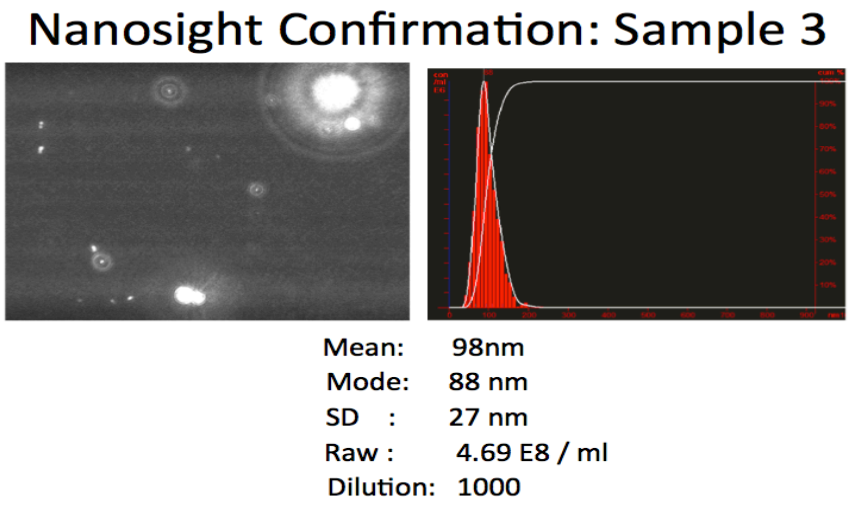


Figure 15 | **A. Graphical bar chart representation of NTA analysis results from the human plasma exosome isolations from 3 participants showing the calculated concentration of particles/ml of plasma and their modal size. B. Nanosight still image of moving particle**

Discussion

From our results we can see that in all three participants our isolation technique produces on average 2.4 x 10^{11} particles per ml plasma. The modal size of particles ranged from 88-96nm in size. This is within the expected size range of exosomes. Additionally sterile buffer alone shows no particles thus all particles seen are a true reflection of sample isolation. We therefore conclude that our isolation technique successfully isolates exosomes from human plasma as evidenced by visually consistent particles on the NTA machine. Of interest, our NTA analysis results show the presence of some larger particles in our sample, though in much smaller numbers.

These larger particles may be microvesicles, plasma proteins, or potentially other subcellular organelles. A potential limitation of NTA is that it is unable to distinguish vesicles from other similarly sized particles, but when our NTA data is taken in combination with EM and western blotting results, it strongly indicates the presence of high quantities of exosomes.

What is the variability in numbers of exosomes isolated from our isolation protocol from human plasma? In addition calculate the internal variability of the NTA machine analysis protocol.

Detailed methods

Human Exosome Isolation

Exosomes were isolated from 81ml of human bloods (18 x 4.5mL BD Vacutainer glass plasma tubes) as described previously up until the final ultracentrifugation step. After the third ultracentrifuge wash step the supernatants from each centrifuge tubes are discarded for final time. Pellets re-suspended in 500 µl sterile PBS from 2 centrifuge tubes and combined to form 1 sample – labelled “Sample 1”. Pellets from the next 2 centrifuge tubes were re-suspended in 500 µl sterile PBS and combined to form 1 sample – labelled “Sample 2”. Pellets from the final 2 centrifuge tubes were re-suspended in 500 µl sterile PBS and combined to form 1 sample – labelled “Sample 3”. The result was 3-exosome samples label 1 to 3.

Nanosight Analysis

To test internal variability of the NTA machine analysis protocol 3 x10µl of exosome sample 1 were diluted in 1ml PBS to give 3 dilution samples. These samples were then analysed on the NTA machine as described in the detailed methods in previous experiment. To test the variability in numbers of exosomes isolated from our isolation protocol from human plasma, 10µ from each exosome sample labelled 1-3 was diluted in 1ml sterile PBS and then analysed on the NTA machine as described in the detailed methods in in previous experiment**.**

Three sample dilutions created as per NTA analysis protocol, resulted in particles concentrations of 4.7 x 10^8, 4.86 x 10^8 and 4.93 x 10^8 particles/ml of sample. The results show a variability coefficient of 2.4% for the NTA analysis. (Table 6, Figure 15).

Variation in NTA machine analysis protocol

Table 6 - Table of NTA analysis results from 3 identical dilutions from the same sample of human plasma exosomes (as per NTA analysis protocol) to calculate the variability coefficient of the NTA analysis protocol. These results show a variability coefficient of 2.4% for the NTA analysis.

Dilution	Concentration (particles /ml of sample)	Standard Error	Modal Size	Standard Error
1	4.7E+8	0.12	73.8	1.5
2	4.86E+8	0.26	76.3	1.2
3	4.93E+8	0.11	98.0	3

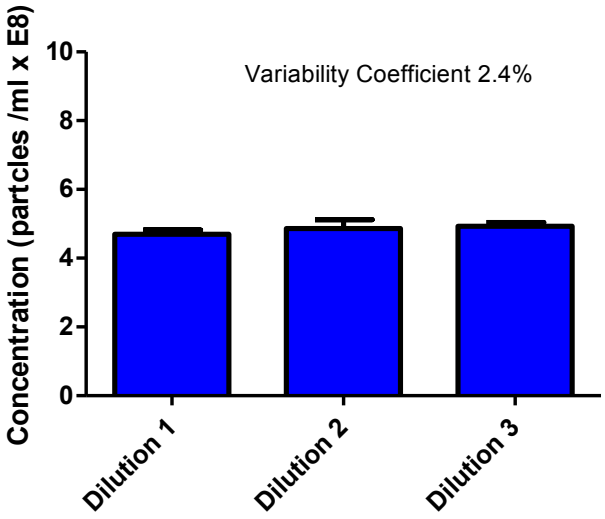


Figure 16 **Graphical bar chart representation of NTA analysis results from 3 identical dilutions from the same sample of human plasma exosomes (as per NTA analysis protocol) to calculate the variability coefficient of the NTA analysis protocol.** These results show a variability coefficient of 2.4% for the NTA analysis.

Variation in Exosome Isolation

3 human plasma exosomes samples were isolated from the same person at the same. We observed Nanosight results showing particle concentrations of 4.93 x 10^8, 4.57 x 10^8, 5.43 x 10^8 per ml of sample. This results in a variability coefficient of 8.6% for the human plasma exosome isolation protocol.

Table 7 - Table of NTA analysis results from 3 human plasma exosomes samples taken and isolated from the same person at the same to calculate the variability coefficient of the human plasma exosome isolation protocol. These results show a variability coefficient of 8.6%.

Sample	Concentration (particles/ml of sample)	Standard Error	Modal Size	Standard Error
Sample 1	4.93E+8	0.11	98	3
Sample 2	4.57E+8	0.17	88.8	8.65
Sample 3	5.43E+8	0.24	78.4	1.03

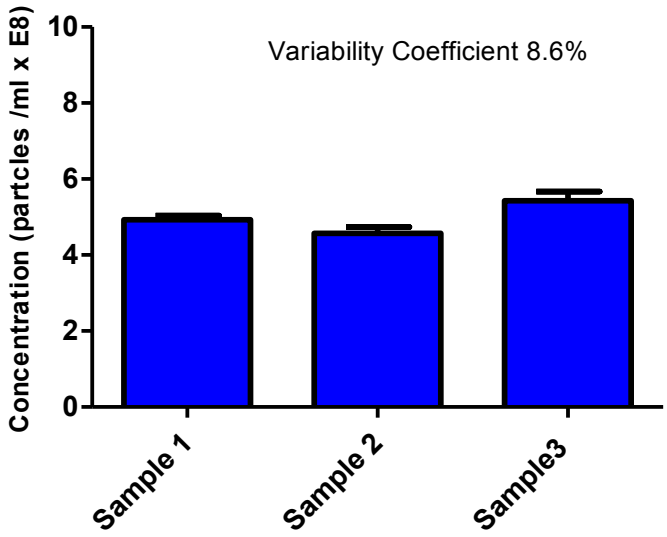


Figure 17 | **Graphical bar chart representation of NTA analysis results from 3 human plasma exosomes samples taken and isolated from the same person at the same to calculate the variability coefficient of the human plasma exosome isolation protocol. These results show a variability coefficient of 8.6%.**

Discussion

Inter-sample variation on taking dilutions for nanosight analysis was within acceptable limits with a variability coefficient of 2.4%. In addition our exosome isolation protocol produced reproducible results within acceptable limits with exosome sample variability coefficient of 8.6%. Typically, coefficients of variation <10% are regarded as being acceptable for assaying variation in clinical samples, thus our assay procedure is acceptable for obtaining reproducible data.

Using the Nanosight what was the optimal storage conditions of exosomes for future analysis and experiment use?

Detailed Method

Samples 1-3 from previous experiment analysed on day 0 as per previous results then split into two. One sample stored at 4°C and one sample stored at -80°C. Both 4°C and -80°C samples then re-analysed 5 days later on the nanosight to see optimal storage conditions.

Results

Three human plasma exosomes samples analysed on day 1 then at day 4 with samples being stored at 4 °C and -80 °C to ascertain optimal storage conditions. Results show storing exosome samples at -80 °C appears to maintain exosome concentrations. (Table 8, Figure 17).

Table 8 - Table of NTA analysis results from three human plasma exosomes sample analysed on day one then at day 4 with samples being stored at 4 °C and -80 °C to ascertain optimal storage conditions. Results show storing exosome samples at -80 °C appears to maintain exosome concentrations.

Sample	Day 0 (particles/ml x10E8)	4 °C (particles/ml x10E8)	-80 °C (particles/ml x10E8)
1	4.93	1.78	4.12
2	4.57	1.65	3.75
3	5.43	2.11	5.17

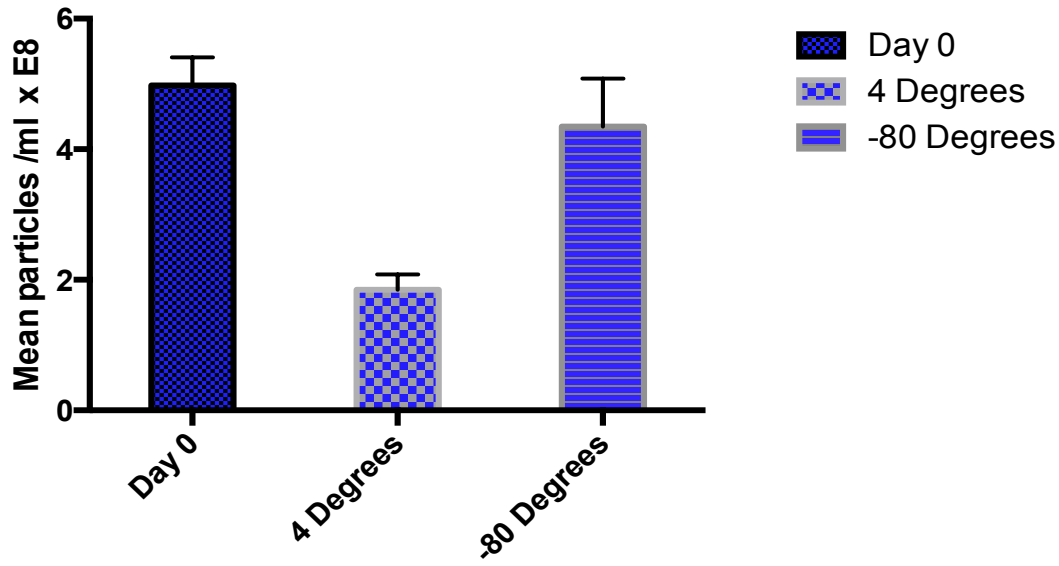


Figure 18 **Graphical bar chart representation of NTA analysis results from three human plasma exosomes sample analysed on day one then at day 4 with samples being stored at 4 °C and -80 °C to ascertain optimal storage conditions. Results show storing exosome samples at -80 °C appears to maintain exosome concentrations.**

Discussion

From the following experiment we conclude the optimal storage conditions for exosome samples were at -80°C, which was consistent with other literature findings[244].

Fluorescence-activated cell sorting (FACS) analysis

Though now confidant that our isolation protocol indeed isolated exosomes from human plasma. We were additionally keen to assess the viability to analyze exosomes through flow cytometric analysis. As evident from our western blotting experiments, protein and biomarker detection in exosomes themselves can be problematic due to the low protein yield and the probable lack of sensitivity of primary antibodies. Although flow cytometry is incapable of directly detecting particles of less than ~300 um including exosomes, an alternative approach is to adsorb exosomes onto small, latex, microsphere beads. This can make biomarker detection easier and thus provide another tool in exosomal analysis. In principle this technique should only detect biomarkers and proteins that lie on the surface of exosomes, so can also provide information as to the location of proteins. In the following experiment we therefore aimed to determine a protocol for human plasma exosome analysis by flow cytometry

and to further confirm the presence of exosomes through our isolation technique through the detection of the known exosomal proteins CD63, HSP70, CD9, CD81.

Specific aims

1. Determine a protocol for human plasma exosome analysis through FACs analysis
2. Confirm the presence of exosomes through our isolation technique through the detection of the known exosomal proteins CD63, HSP70, CD9, CD81

Determine a protocol for human plasma exosome analysis through FACs analysis

Detailed methods

Human Exosome Isolation

Human plasma exosomes were isolated using the same protocol used in experiment 3.1.2.2.

FACS analysis protocol

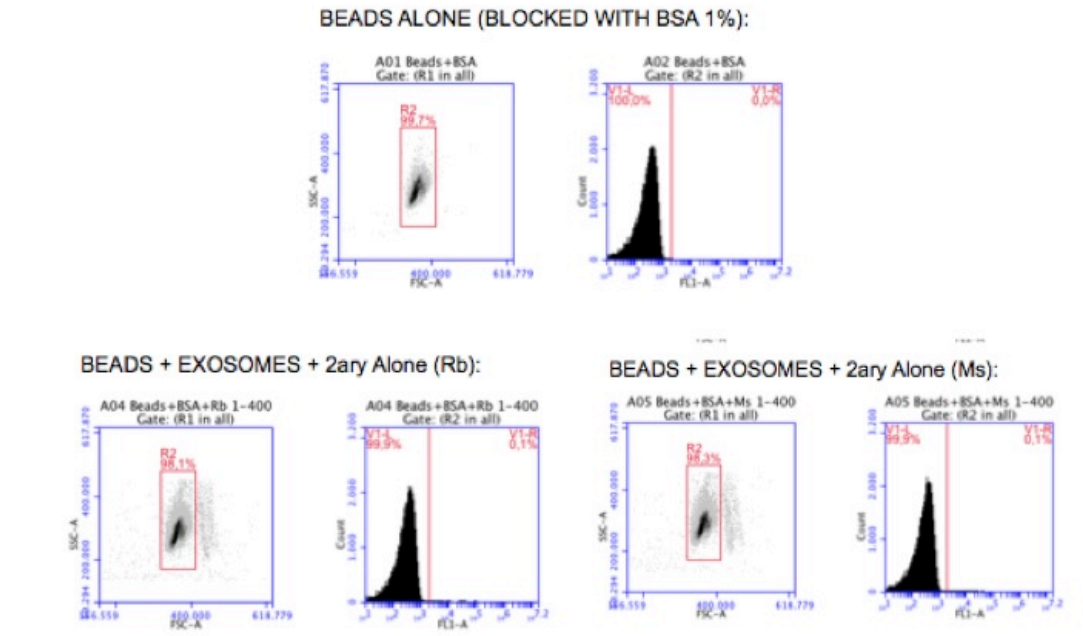
Exosome samples were analysed on NTA analysis machine as per detailed protocol in previous experiments to calculate number of exosome per ml of sample. After Nanosight quantification of each exosome sample, the volume of sample needed to coat the microspheres with at least $2x10^9$ exosomes was calculated. Exosome samples were then cleared of macromolecular IgG complexes. The volume calculated from Nanosight analysis (previous step) was then added to PBS solution and 20 µl of G protein sepharose beads (which binds IgG), to make up a 1ml sample. This sample was then rotated at 4 °C for 60 min and centrifuged 9000 rpm, 4 min, 4 °C. The supernatant containing cleared exosomes was used for binding to the latex aldehyde microspheres. Cleared exosomes were taken into a new tube and 5µl of aldehyde beads (Aldehyde/Sulphate Latex Beads, 4% w/v, 4µm Molecular Probes)) were added then incubated for 1 h at 4 °C with rotation for binding of the exosomes to the microspheres. The sample was then centrifuged at 9000 rpm, 4 min, 4 °C. The supernatant was removed and 1ml PBS added. Centrifugation and removal of supernatant was repeated a further two times as washing steps. The exosome-coated microspheres were re-suspended and blocked in 1 ml of 1% BSA/PBS + 0.1% NaN3 (blocking buffer). This was then left to rotate at 4 °C for 1 hour. 100µl aliquots of the blocked beads were then transferred into new tubes. These aliquots were then incubated with different primary antibodies for 1 h at 4°C for the detection of specific

markers: 1:100 (HSP70, CD63, CD9, CD 81). The microsphere beads, stained with primary antibody, were washed three times through a process of centrifugation at 9000 rpm, 4 min, 4 °C in blocking buffer. After the third wash the samples were re-suspend in 100µl blocking buffer and incubated with the appropriate fluorescent secondary antibodies for 40 min at 4°C

- HSP70 (1:200 Anti-Mouse Alexa 488).
- CD63 (1:200 Anti-Rabbit Alexa 488).
- CD9 (1:200 Anti-Mouse Alexa 488).
- CD 81 (1:200 Anti-Rabbit Alexa 488).

Once incubated with the secondary antibody, the exosome-coated beads were washed 3 times by centrifugation and finally re-suspended in 100µl blocking buffer, working in a dark environment to prevent fluorescence photo bleaching. The optimal dilutions for all antibodies were titrated, after a series of experiments with different titres of primary and secondary antibodies. The microsphere beads were then analysed on a flow cytometer (Accuri C6, BD Biosciences) using the software Cflview PlusAnalysis (BD Biosciences). Each aliquot was aspirated into the flow cytometer and results were presented graphically. Results were presented as histogram plots, against control experiments using beads that were blocked with BSA, in the absence of exosomes. On each experiment, control samples with no exosomes were used.

The results of flow cytometry analysis of human plasma exosomes show the detection of classical exosomal proteins CD63, CD81, CD9 and HSP70. (Figure 18)



A

B

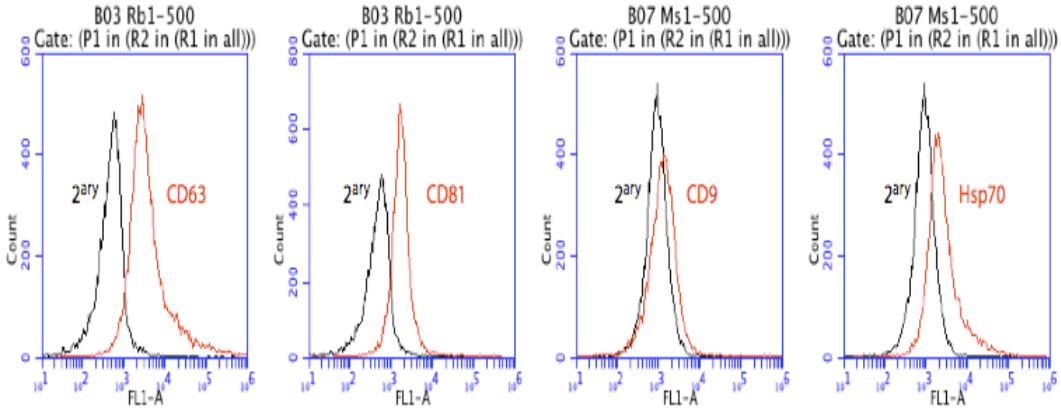


Figure 19 **Flow cytometry analysis of human plasma exosomes. Beads alone as well as beads with exosomes and secondary antibody show no increase in signal.** The subsequent results show the detection of classical exosomal proteins CD63, CD81, CD9 and HSP70. “FL1-A” indicates fluorescence channel 1, in which fluorescence of the secondary antibody was detected. “Count” on the x-axis represented the number of beads detected with that fluorescence intensity. The same sample was stained with the primary antibody indicated (red curve), or secondary antibody alone (“2ary”, black).

Discussion

From the following experiment we demonstrate a viable protocol of exosomal biomarker and protein content analysis via flow cytometry. We were able to further confirm the successful isolation of human plasma exosomes from our protocol by the detection of the classical exosomal proteins CD63, CD9, HSP70 and CD81. Even the tetraspanin proteins, CD9 and CD81 that were not detectable via western blotting were detected by our flow cytometry analysis. These results therefore support our hypothesis that flow cytometric analysis may be a more sensitive method for biomarker analysis of human plasma exosomes - certainly for those biomarkers or proteins that reside on the surface of the exosome.

MicroRNA analysis

Establish the presence of, and isolate microRNA from human plasma exosomes.

The extent to which plasma miRNA is contained within exosomes is still controversial. Some studies suggest that exosomes contain the majority of plasma miRNA[197], while others find plasma miRNAs are mainly in argonaute complexes [198]. However, numerous studies have demonstrated the ability of exosomes to transfer miRNA to other cells[195,199] and miRNA have been implicated in both ischaemia reperfusion injury and heart failure(ref). The ability to regulate specific miRNA and their function could present a novel cardio-protective strategy. The optimal isolation method of miRNA from human plasma exosomes is yet to be established. We aimed to establish an isolation protocol for miRNA from human plasma exosomes and explore their potential role in cardio-protection in RIPC.

Specific aims

1. Isolate miRNA from human plasma exosomes.

Detailed Methods

Human plasma exosomes were isolated 72ml blood from recruited participants as per previously described and exosome pellets re-suspended in the initial experiments 500µl PBS. After various optimization experiments human plasma exosomes, isolated by ultracentrifugation, were finally re-suspended directly in 250µl miRVana homogenization buffer to improve quality and yield of RNA. miRNA isolation was

carried out at the Centre of Cardiovascular Genetics and thus is described briefly for the purpose of this thesis. miRNA isolation was carried out using the miRVana kit (Ambion, Austin, TX, USA). The RNA was further purified with 4 ethanol washes and RNA pellet re-suspended in 20 µl of distilled H_2O. Finally RNA was purified further with DNAse I treatment. miRNA expression measurements were carried out using real time-PCR.

Expression levels for miR-16, snoU6 and RNU6B were first tested for. miR16 has been described as a surrogate marker of bulk exosome release[247]. snoU6, a small nucleolar "guide" RNA molecule that primarily guides chemical modifications of other RNAs. Lastly RNU6B is a small nucleolar RNA and common normalization control for miR expression. These two endogenous reference RNA's were used to normalize the amount of target miRNA (miR-16). In addition GAPDH, an mRNA known to be expressed in exosomes[195] was quantified with a view to using its expression on exosomes as an alternative-normalizing gene. This was carried out using a High Capacity RNA-to-cDNA kit. Ubiquitin C expression was tested to measure for relative expression. Runs were conducted at 40 cycles and 1µg OD cDNA was used for each 20 µl reaction. Reactions were performed in triplicates.

Results

We observed a miR signal from human plasma exosome samples for miR-16, snoU6, Ubiquitin C and GAPDH. RNU6B was not expressed at all in our samples. Results show SnoU6 as a better normalization control for our human exosome samples and thus this was used as a reference to calculate $2^{-}\Delta\Delta Ct$ for miR16 and GAPDH. (Table 9 Figure 19)

Table 9 - Table of Average Ct readings (relative measure of the concentration of target in the PCR reaction) for miR-16, RNsnoU6, RNU6B, GAPDH and Ubiquitin C. $2^{-}\Delta\Delta Ct$ compared to RNsno16 were also calculated for miR-16 and GAPDH.

miRNA	Average Ct	Average $2^{-}\Delta\Delta Ct$ compared to RNsno16
miR-16	29.08	1.025
RNsnoU6	35.58	NA
RNU6B	NA	NA
GAPDH	34.01	1.028
UBC	36.24	NA

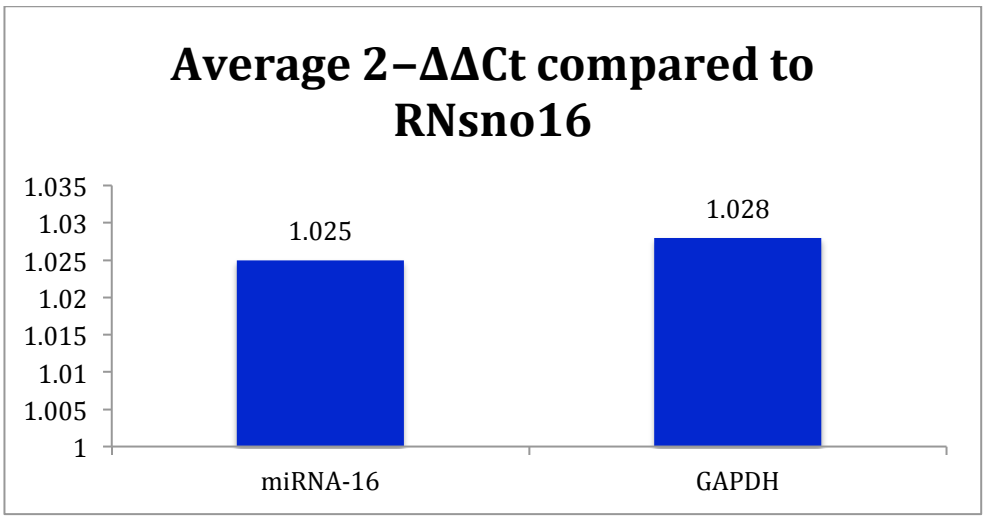


Figure 20 | Graph of 2−ΔΔCt compared to RNsno16 for miR-16 and GAPDH.

Discussion

Though in small quantities we were able to successfully isolate microRNA from our human plasma exosome samples. We observed signals from human plasma exosome samples for miR-16, snoU6 and GAPDH. UBC was expressed at close enough levels to be used as normalization control for GAPDH. SnoU6 is a better normalization control for our human exosome samples while RNU6B was not expressed at all in our samples. These results provide a protocol for further microRNA experiments on human plasma exosomes. Experiments comparing the microRNA content of RIPC and control exosomes can now be carried out.

CHAPTER 4

Results

A comparison of RIPC and Control Exosomes

Introduction

As discussed earlier the mechanism of cardio-protection conferred through remote ischaemic preconditioning is not fully understood. Humoral factors are proposed to play a key role, yet the identification of the precise humoral factors have been challenging. Indeed it may be several humoral factors working in conjunction that lead to the cardio-protection seen through RIPC. In the following experiments we look to see what the effect of remote ischaemic preconditioning has on human plasma exosomes, both in terms of their quantity as well as content.

Specific Aims

1. Compare and quantify exosomes from human plasma taken after remote ischaemic preconditioning (RIPC exosomes) with exosomes taken from normal human plasma (control exosomes) by Nanosight analysis
2. Compare the protein content of RIPC exosomes with control exosomes by Western Blot analysis.
3. Compare the protein content of RIPC exosomes with control exosomes by flow cytometry analysis.
4. Compare the miRNA content of RIPC exosomes and Control exosomes

Results

Compare and quantify exosomes from human plasma taken after remote ischaemic preconditioning (RIPC exosomes) with exosomes taken from normal human plasma (control exosomes) by Nanosight analysis

Detailed Methods:

- *Exosome Isolation*

  6 participants were recruited into this experiment. Each participant had 72ml of human bloods (16x 4.5mL BD Vacutainer glass plasma tubes) before (control blood) and after a RIPC stimulus of 3 cycles of 5 minutes arm cuff inflation followed by 5 minutes deflation (RIPC blood). Exosomes were then isolated from control blood and RIPC blood as described previously up until the ultracentrifugation steps while ensuring both are kept separate. 3 x10ml of control human platelet free plasma put in 3 ultracentrifuge tubes and similarly 3 x10ml of RIPC human platelet free plasma put in the last 3 ultracentrifuge tubes. The plasma is then centrifuged at 100,000 x g for 90 min, 4 °C with a SW-41 rotor. The supernatant is discarded leaving ~ 10µl and then re-suspend in 8 ml PBS. (Ensuring different pipettes used for control and RIPC samples). A further ultracentrifuge wash step undertaken at 100,000 g 60 min, 4 °C in Beckman Optima L in SW-41 for 1 hr. Supernatant discarded leaving ~ 10µl and re-suspend in 8 ml PBS. Last ultracentrifuge wash step undertaken at 100,000 g 60 min, 4 °C in Beckman Optima L in SW-41 for 1 hr. Exosome pellets (non–visible) from the 3 control ultracentrifuge tubes were re-suspended in 1ml sterile PBS combined to form 1 control exosome sample. Exosome pellets from the 3 RIPC ultracentrifuge tubes were re-suspended in 1ml sterile PBS combined to form 1 RIPC exosome sample. Each exosome Control and RIPC exosomes, divided into 100µl aliquots, labelled and stored at -80°C for storage for further experiments.

- *Nanosight analysis*

  Both control and RIPC exosomes were analysed on the Nanosight using the methods described in previous experiments. Participants 4 and 5 had to be diluted further to 1:400 and 1:200 concentrations respectively to comply with nanosight manufacturer recommendations of particles per screen for optimal analysis.

Results

We observed in all 6 participants a significant rise in exosome concentration (Table 10 Figure 20):

Table 10 - Table of NTA analysis results from 6 human plasma exosomes samples pre and post RIPC. Participant 4 and 5's exosome samples had to have further dilutions (1:400 and 1:200) as compared to the other samples (1:100) to achieve NTA software recommendation recommendations for number of particles on the screen to achieve optimal analysis. Results show in each participant a rise in exosome numbers after RIPC.

Participant	Control Exosomes (particles/ml sample x10E8)	Standard Deviation	RIPC Exosomes (particles/ml sample x10E8	Standard Deviation
1	3.64	0.46	4.87	0.46
2	2.87	0.30	3.36	0.18
3	2.59	0.41	7.78	0.72
4 (1:400)	7.25	0.38	12.99	2.14
5 (1:200)	2.39	0.43	9.25	0.80
6	3.41	0.46	5.26	0.51

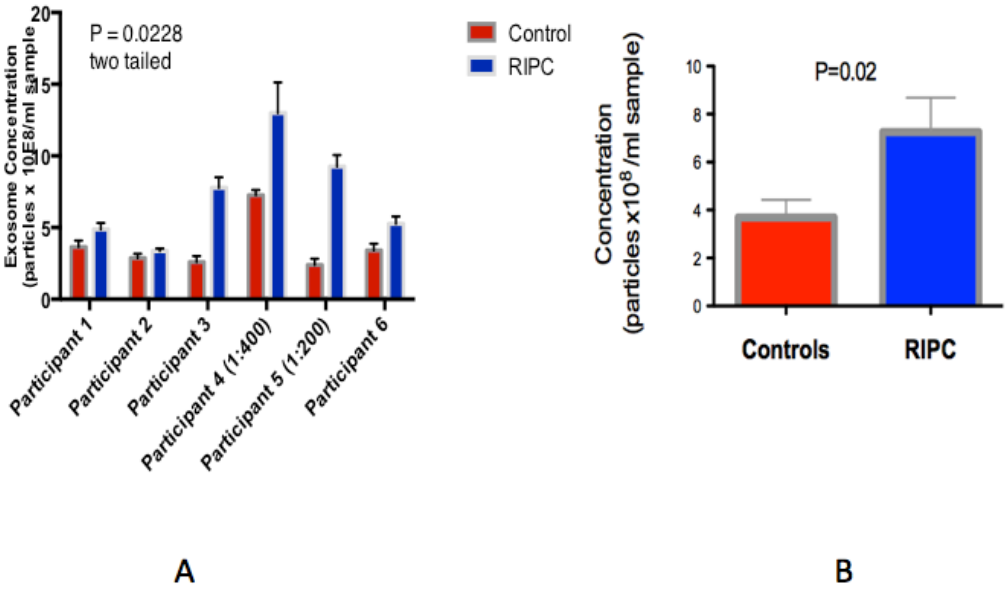


Figure 21 **Graphical bar chart representation of NTA analysis: A. Results from 6 human plasma exosomes samples pre and post RIPC.** Participant 4 and 5's exosome samples had to have further dilutions (1:400 and 1:200) as compared to the other samples (1:100) to achieve NTA software recommendations for number of particles on the screen to achieve optimal analysis. B. Pooled analysis of all participants. Results show a significant rise in exosome numbers (P=0.02 two tailed) after RIPC.

Discussion:

The results show interestingly that remote ischaemic preconditioning of three cycles of forearm cuff inflation and deflation lead to a rise in plasma exosome concentration. This is seen consistently through all the participants. This trend is also seen in the parallel rat exosome experiments carried out within our research group. The rise in exosome concentration may indicate a role for exosomes in the cardio protection that is conferred through RIPC.

At what time point do exosomes concentration rise post RIPC stimulus?

As discussed previously, current convention for the remote ischaemic preconditioning stimulus stands at 3 cycles of 5 minutes forearm cuff inflation followed by 5 minutes of forearm cuff deflation. Though there have been no studies to date that confirm this as

the optimal RIPC protocol. We carried out a small experiment to gauge at which time point resulted in the corresponding rise in exosome concentrations seen previously.

Detailed Methods:

3 participants were recruited; each participant had 9ml of human bloods (2x 4.5mL BD Vacutainer glass plasma tubes) taken before (control blood) the RIPC stimulus. Then a further 9ml of human blood 30 seconds after the last of 3 RIPC cuff inflations, then finally a further 9ml of human blood 5 minutes after the final RIPC cuff inflation. Exosomes where then isolated as per previously described from 4ml of plasma per group (Control, 30 seconds post RIPC and 5 minutes post RIPC). Exosomes were then analysed on the NTA analysis machine as per previously described protocol.

Results:

Peak rise in exosome concentrations occur only after the last 5 minutes of cuff deflation (reperfusion) in the 3 cycles of RIPC protocol. (Table 11, Figure 21)

Table 11 - Exosome Concentrations at different time points (control, 30 seconds post RIPC and 5 minutes post RIPC). Exosomes isolated from 4ml plasma and pellet suspended in 1ml PBS. 1:100 dilutions taken for nanosight analysis.

PARTICIPANT	CONTROL		30 SECS POST RIPC		5MINS POST RIPC	
	CONC (particles/ml)	SD	CONC (particles/ml)	SD	CONC (particles/ml)	SD
Participant 1	2.80E+08	0.13	4.09E+08	1.09	8.54E+08	0.64
Participant 2	5.40E+08	0.22	6.11E+08	0.54	6.76E+08	0.41
Participant 3	3.55E+08	0.31	4.68E+08	0.21	1.10E+09	0.32

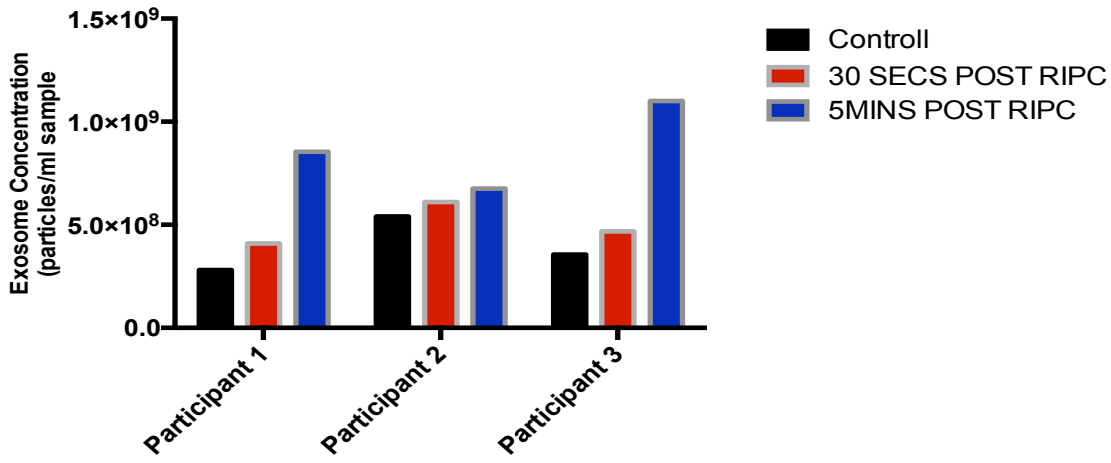


Figure 22 **Exosome Concentrations at different time points (control, 30 seconds post RIPC and 5 minutes post RIPC). Exosomes isolated from 4ml plasma and pellet suspended in 1ml PBS. 1:100 dilutions taken for nanosight analysis. Results suggest the peak rise in exosome concentrations occur only after the last 5 minutes of cuff deflation (reperfusion) in the 3 cycles of RIPC.**

Discussion

Our results suggest a peak rise in exosome concentrations occur only after the last 5 minutes of cuff deflation (reperfusion) in the 3 cycles of RIPC protocol. These results may provide support to the convention of using 3 cycles of 5 minutes of cuff inflation and deflation. Though beyond the scope of this project further experiments should be conducted to ascertain the precise point of rise in exosome concentration following RIPC. Could indeed one cycle 5 minutes of cuff inflation and deflation be enough to produce a similar response? It would also be interesting to determine the response in human plasma exosome concentration to further cycles of RIPC.

Compare the HSP70 and CD63 content in RIPC exosomes with control exosomes by Western Blot analysis

Though confident that RIPC produced a rise in human plasma exosome concentration from our NTA analysis it was important to confirm an increased presence of exosomes in our RIPC samples when compared to our control and not other particles such as apoptotic bodies. We therefore took each participant RIPC and control samples from experiment 4.1.1 and analysed and compared the concentrations of HSP70 and CD63. If indeed RIPC produced a greater concentration of human plasma exosomes therefore

one would expect a greater concentration of exosomal protein markers HSP70 and CD63 in the RIPC sample

Detailed Methods:

The 6 RIPC and Control human exosome samples from experiment 4.1.1 were used for this experiment. For each sample the protein concentration from the extraction and thus protein concentration per ml of each sample was ascertained using the protein assay protocol used in experiment 3.1.2. The protein assay results were then used to calculate the volume of sample need to load equal protein concentrations of RIPC and control sample on to SDS PAGE western blot gels. Participant 4's samples were not put forward for further analysis for fears of impurity given the large protein concentration when compare to the other samples. Exosome sample preparation and western blot analysis was carried out using the same protocol as previously described. Samples were probed for HSP70 and CD63. Density of bands were then analysed to quantify signals using Image J software.

Results:

Here we detail the protein concentrations of each RIPC and control exosome isolation sample from all 6 participants. Of note we observe large amount of protein in participant 4's control sample when compared to the other samples. This raises the possibility of impurity and thus this participant's samples not taken forward for western blotting.

Table 12 - Table of protein concentration (µg/ml) of exosome isolation samples, RIPC and Control from the 6 participants. Note large amount of protein in participant 4's control sample when compared to the other samples, thus not taken forward for western blotting for fear of impurity.

Participant	Protein from total control extraction (µg/ml) sample	Protein from total RIPC extraction (µg/ml) sample
1	287.86	328.36
2	98.45	124.62
3	69.16	108.42
4	2767.72	883.53
5	651.12	527.13
6	586.32	699.72

Our western blot analysis demonstrates demonstrate greater concentration of HSP70 and CD63 consistently in the RIPC samples when compared to the corresponding control sample. (Figure 22)

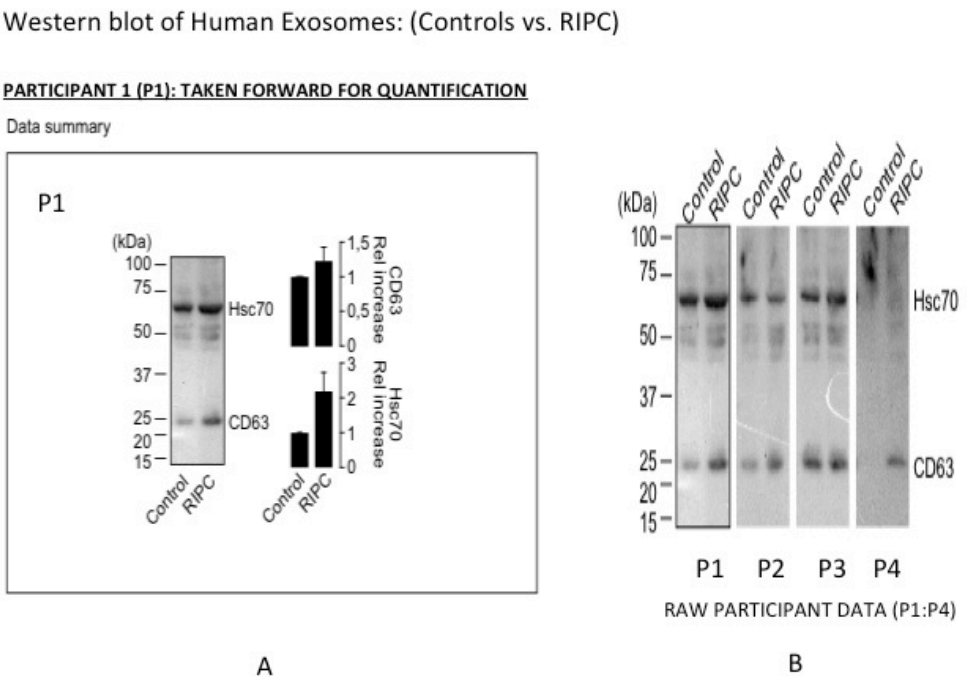


Figure 23 **Western blot analysis of HSP70 and CD63 for comparison between human RIPC and control exosome samples in 4 participants (P1:P4). A. Participant 1 (P1) taken forward for quantification. Results demonstrate greater concentration of HSP70 and CD63 consistently in the RIPC samples. B. Raw data for all participants shown.**

Discussion:

Our results show that RIPC human plasma exosome samples contain more HSP70 and CD63 than control human plasma exosomes. This therefore confirms our NTA analysis findings that RIPC increases circulating human plasma exosome concentrations. This raises the question whether this rise in exosomes may play a role in the cardio protection conferred by RIPC.

Compare RIPC exosomes with control exosomes by Western Blot analysis for known cardio-protective signalling proteins

The pro-survival protein kinases of the RISK pathway have been shown to confer cardio-protection against ischaemia reperfusion injury [48]. RIPC has also been shown to protect via the RISK pathway[5,9,248]. Traditionally these pro-survival kinases are activated through G protein coupled receptors (GPCR), however we tested the hypothesis that human plasma exosomes, particularly RIPC exosomes, may contain these active kinases and therefore confer cardio protection directly when released in to the cell.

Detailed Methods

Further human participants were recruited for exosome isolation. All participants underwent RIPC protocol and exosome isolation protocol as described previously. Human exosome samples, both RIPC and control were analysed through SDS PAGE western blot analysis as previously described. RIPC and exosome samples were probed for the following pro-survival protein kinases of the RISK: P13K, MEK 1/2, AKT, ERK1/2 using their primary antibodies (1:100 concentration)

Results:

Our results show that western blot analysis on RIPC and Control human plasma exosomes did not detect the presence of known pro-survival kinases of the RISK pathway. (Table 13)

Table 13 - Table showing the results of western blots analysis of human RIPC and control plasma exosomes for known pro-survival kinases P13K, MEK 1/2, AKT, ERK1/2. Pro-survival kinases were not detected in our RIPC and control human plasma exosomes.

Pro-survival kinase	Detected in Control Exosomes?	Detected in RIPC Exosomes?
P13K	No	No
MEK ½	No	No
AKT	No	No
ERK ½	No	No

Discussion:

Despite multiple attempts, we could not demonstrate the presence of pro-survival kinases directly in human plasma control or RIPC exosomes. This may simply be a limitation of working with samples that contain such a small amount of protein. However if the result is true, and indeed pro-survival kinases are not directly located in human plasma exosomes (especially RIPC exosomes), there may be other signalling proteins or growth factors within human plasma exosomes that may work at a higher level in the RISK pathway, and directly on the GPCR. Indeed HSP70 itself has been shown to have cardio-protective properties[249–251], however this protein is seen in both RIPC and control exosomes. This could possibly explain a mechanism for cardio-protection through plasma exosomes in general, however would not explain an exosomal mechanism for the cardio-protection conferred through RIPC as HSP70 is seen in both RIPC and control exosomes

Compare RIPC exosomes with control exosomes by flow cytometry for known cardio-protective signalling proteins

As described previously our experiments suggest flow cytometry may provide a more sensitive tool for the detection of biomarkers in exosomes. We therefore used FAC's analysis method to attempt to detect the same pro survival kinases P13K, MEK 1/2, AKT, ERK1/2 in our RIPC and control exosomes.

Detailed Methods:

The RIPC and control human plasma exosome samples isolated in the previous experiment were also used in this experiment. FACS analysis was carried out using the protocol as described previously using the same concentrations for primary (1:100) and secondary antibody (1:200).

Results

Our results show that FACS analysis on RIPC and Control human plasma exosomes did not detect the presence of known pro-survival kinases of the RISK pathway. (Table 14)

Table 14 - Table showing the results of FACS analysis of human RIPC and control plasma exosomes for known pro-survival kinases P13K, MEK 1/2, AKT, ERK1/2. Pro-survival kinases were not detected in our RIPC and control human plasma exosomes.

Pro-survival kinase	Detected in Control Exosomes?	Detected in RIPC Exosomes?
P13K	No	No
MEK ½	No	No
AKT	No	No
ERK ½	No	No

Discussion:

We could not demonstrate the presence of pro-survival kinases on the surface of human plasma control or RIPC exosomes through FACS analysis.

CHAPTER 5

Are human RIPCS plasma exosomes cardio-protective against ischaemia reperfusion injury?

Introduction

A role for exosomes in cardio-protection has not been fully established though as described previously, preliminary work by various groups implicate that exosomes from various cell lines indeed may have some involvement in cardio-protection. In this experiment we hypothesis that the cardio-protection conferred through RIPC is mediated through circulating plasma exosomes. We therefore tested the hypothesis that circulating RIPC plasma exosomes will confer cardio-protection in an invitro model of ischaemia reperfusion injury while control plasma exosomes will not.

Aims

Establish if RIPC exosomes are cardio-protective in a an in-vitro model of ischaemia reperfusion injury using rat cardiomyocyte cells

Methods

The detailed methods for this experiment are described in the methods and material chapter. The experiment was carried out in collaboration with Jose Vincencio and Jessica Kearney from the Hatter Cardiovascular Institute, who helped with the rat myocyte isolation and in-vitro simulated ischaemia reperfusion testing.

Results

Our model of simulated ischaemia reperfusion was confirmed to work with an increase in cardiomyocyte cell death from 13 ± 3% to 59 ± 7% (n=5, P<0.001). Both the addition of 5 µl of RIPC exosomes or control exosomes at reperfusion conferred significant cardio-protection and reduced cardiomyocyte cell death to 17 ± 3% and 17 ± 4% respectively. A dose response experiment was undertaken with the addition of serial dilutions of RIPC and control exosomes at reperfusion. Our results show that the addition of 0.05µl and above of both isolated control and RIPC plasma exosomes confer cardio-protection against simulated our ischaemia reperfusion. (Table 15, Figure 23)

Table 15 - Table showing the results of survival analysis of invitro model of ischaemia reperfusion injury using rat cardiomyocytes. Results show % cell death in normoxia, untreated samples. Then with the addition at re-oxygenation of Insulin as a positive control and finally serial dilutes of control and RIPC plasma exosomes (5µl-0.0005µl, n=5).

Treatment at Re-oxygenation (n=5)		Average % Cell Death
Normoxia		10.68
Untreated		53.56
Insulin		14.26
5 µl exosomes	Control	17.37
	RIPC	17.45
0.5 µl exosomes	Control	24.14
	RIPC	19.40
0.05 µl exosomes	Control	31.28
	RIPC	28.23
0.005 µl exosomes	Control	57.33
	RIPC	40.58
0.0005 µl exosomes	Control	71.35
	RIPC	64.78

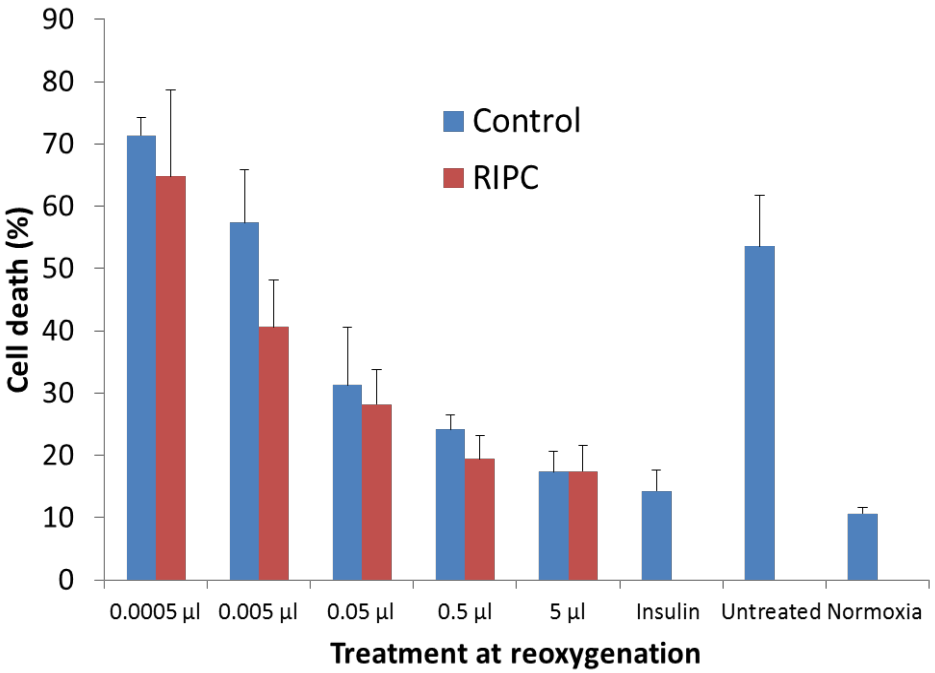


Figure 24 **Graph showing the results of survival analysis of invitro model of ischaemia reperfusion injury using rat cardiomyocytes. Results show % cell death in normoxia, untreated samples. Then with the addition at re-oxygenation of Insulin as a positive control and finally serial dilutes of control and RIPC plasma exosomes (5µl-0.0005µl, n=5).**

Discussion

Our results demonstrate that plasma exosomes are cardio-protective against IR injury irrespective if they are control or RIPC plasma exosomes. The protection conferred by plasma exosomes are comparable to Insulin, a known cardio-protective agent, used here as our positive control. These results suggest that plasma exosomes in general are cardio-protective and may not have a mechanistic role in the cardio-protection conferred through RIPC. There is some divergence in the degree of cardio-protection with the addition of 0.005µl of control and RIPC plasma exosomes. At this concentration RIPC exosomes still provide some degree of cardio-protection yet the control plasma exosomes do not. This however may simply be due to the lower concentration of exosomes in the control sample. We know from our previous experiment that RIPC exosome samples have a higher concentration of exosomes. Conversely this could also provide a tentative mechanism linking the cardio-protection conferred through RIPC with plasma exosomes. Does the effect of increased plasma levels of exosomes seen after RIPC help with cardio-protection, rather than a plasma exosome with a differing content?

CHAPTER 6

Discussion

Exosomes are increasingly thought to play a novel role in intracellular communication. The primary aim of this thesis was to begin to explore the potential role for plasma exosomes in the mechanism of RIPC. Could plasma exosomes provide a mechanistic explanation for why the brief and intermittent ischaemia of a distant organ confers cardio-protection against IR injury?

To explore this it was imperative to first establish protocol for isolating human plasma exosomes. At the time of embarking on this project exosomal research had largely been carried out using exosomes isolated from the supernatants of cultured cells. The optimal method of plasma exosome isolation, purification and characterization was subject to continuing investigation and is not straightforward for a number of reasons. Firstly, human plasma is complex containing erythrocytes, platelets, lymphocytes and monocytes etc. as well as being exposed to endothelial and other cell types, thus the source of exosomes in plasma still has a degree of uncertainty. The vast majority of exosomes in human plasma are thought to arise from platelets and erythrocytes. The most commonly used technique for exosome isolation is a process of serial differential centrifugation and ultracentrifugation [244]. This process relies on the physical properties of exosomes in terms of their size and sedimentation coefficient. Some protocols include a step in which exosomes are separated on a density gradient according to their buoyancy, although this adds a further degree of complexity and potential variability to the protocol. It is thought that ultracentrifugation can cause plasma exosomes to aggregate together as a consequence of their protein and lipid rich coating. This tendency for aggregation can significantly reduce the yield from isolation through ultracentrifugation, and can potentially cause co-precipitation of non-exosomal proteins that are trapped in the aggregates. Recognizing this challenge it was only in

late 2013 that Witwer and colleagues published their paper attempting to standardize sample collection, isolation and analysis in extracellular vesicle research[252].

The volume of human blood available of exosome isolation was also a limiting practical factor. In fact at the beginning of this project, the one paper that had described isolation of plasma exosomes from human blood, had isolated plasma exosomes from a significantly larger volume (400-600 ml) of blood than was available to our group (72ml). The limitation was largely due to the ethical constraints of obtaining much larger blood volumes from human participants as well as the practical challenge of persuading participants in providing such. Additionally, the Eppendorf centrifuge system that was available for use in our laboratory would only allow centrifugation of 60ml of plasma at a time to produce platelet free plasma and there was a similar limitation in volume with the ultracentrifuge. Isolating exosomes from a greater quantity in blood would therefore involve further centrifugation steps, 60ml of plasma at a time, necessitating storage of plasma in ice till it could be then also centrifuged. Our concern with this process was that this waiting time would potentially cause the platelet component of the plasma that is a rich source of exosomes to release exosomes and contaminate the sample. Nonetheless, for research in this field to remain viable within the scientific community, protocols must be devised to allow the isolation and purification of plasma exosomes from a small sample of plasma.

After multiple iterations of our isolation protocol of differential centrifugation and ultracentrifugation we were able to establish a protocol for isolation of plasma exosomes from human blood. The isolation of exosomes was confirmed through the presence of cup shaped vesicles by electron microscopy, of the expected size range (50-100nm diameter). Though spherical in solution, the cup shape is thought to be due to the vesicles collapsing during the drying procedure for electron microscopy.

Our next challenge was to produce exosome samples that we felt were pure enough to take forward for further experiments. We embarked on this through an experiment of isolating human plasma exosomes and putting them through a range of one to five serial ultracentrifugation washing steps. Albumin is the most abundant protein in plasma. We therefore used albumin as a surrogate marker for impurity and each sample of human plasma exosome was analyzed through western blotting. We were able to demonstrate that 3 ultracentrifuge-washing steps provided the best balance of purity versus exosome quantity. Although exosomes were undoubtedly more pure after 4 ultracentrifuge steps, the yield was unacceptably low, and would make subsequent analysis impossible. We acknowledge that this method is certainly not perfect, and that

there will still be significant contaminating protein, however there remains no perfect procedure to isolate pure exosomes, nor consensus on what is the optimal procedure[252]. Subsequent groups have used alternative methods to achieve greater purity. These methods have involved affinity purification using exosome membrane antigens or sucrose density gradients separating vesicles based on their buoyant densities. Kalra and colleagues attempted to evaluate these strategies of exosome isolation and concluded that the density gradient method of exosome isolation yielded the more pure samples from human plasma. However they also acknowledge that the density gradient method was laborious and cannot be effectively used yet in the clinical setting for biomarker analysis[253]. Affinity purification typically results in relatively high purity of exosomes, at the expense of yield. Furthermore, the method requires the use of one or more antibodies against specific marker proteins, and therefore the analysis is restricted to those exosomes that express the marker proteins. A number of easy-to-use and inexpensive commercial kits are also available for exosome isolation such as ExoQuickTM (System Biosystems) and Total Exosome Isolation (Life Technologies). The exact methods by which these kits isolate exosomes are not disclosed by their respective companies, but involve selective precipitation by a proprietary polymer. Though used by various groups and indeed these products do successfully isolate exosomes, the purity of the sample being produced is increasingly being questioned[246] . Pilot studies in our laboratory indicated that while yields of “exosomes” were high, the purity was low, as assessed by the appearance of bands by Western Blotting. As yet there is still no consensus amongst the scientific community how to best overcome the challenge of isolating exosomes human plasma in sufficient quantity and purity[252].

Having established a protocol for human plasma exosome isolation that satisfied our requirements for purity and yield, we then went on to probe the samples for known protein markers of exosomes: HSP70, CD63, CD9 and CD81. HSP70 and CD63 were consistently found in exosomes from the plasma from all participants. Interestingly, though classically in literature the molecular weight of CD63 in exosomes is described as 53 kDa. We consistently detected a signal at 26 kDa both in human plasma exosomes but also in concurrent work carried out on rat plasma exosomes. On further review, we ascertained that the molecular weight of CD63 protein itself is 26 KDa, but that glycosylation typically alters its size appearance by Western Blotting. It is not clear why CD63 ran at its native size in our experiments. The other typical tetraspanins tested, CD9 and CD81 were consistently undetectable in our samples. This again was consistent with the findings of other experiments within our group working on rat plasma exosomes. Upon further review difficulties with western blotting with exosomes are not uncommon and more so with plasma. Primarily difficulties arise due to the low

yield of protein and insensitivity of the primary antibodies with variability of success between differing companies producing the primary antibody. Alternatively, the exact complement of tetraspanin molecules may vary between exosomes of different sources, and CD9 and CD81 may not be present at high concentration in plasma exosomes. As this experiment was primarily designed to validate that we had successfully isolated plasma exosomes, the successful identification of HSP70 and CD63, in combination with other data (nanoparticle tracking analysis and EM – see below), was taken to be sufficient. We did not feel it necessary to further investigate using different primary antibodies of CD9 and CD81 from other companies.

The ability to quantify exosome concentrations was critical in order to test our hypothesis that human plasma exosomes had a mechanistic role in the cardio-protection conferred through RIPC. As discussed previously, for many years a reliable methodology of exosome quantification had not been developed and accepted. At the start of this project a new apparatus manufactured by Nanosight which was able to perform nanoparticle tracking analysis (NTA), had entered the market. Its manufacturers claimed the ability to accurately visualize and quantify particles in liquids from 10-2000nm in diameter. Based on the principle that the rate of Brownian movement of nanoparticles in a solution is related to their size and using the Stokes-Einstein equation and that the rate of particle movement is related to a size/sphere diameter of the particle, the NTA machine and its computer software provided allows for the calculation of number of particles in a given solution and the distribution of particles in relation to size of particles. Movement is also dependent on temperature, so temperature is maintained at 37°C, and is monitored and corrected for.

Using the NTA machine equipment we were able to re-affirm the successful isolation human plasma exosomes through visually demonstrating particles consistent within the exosomal range (50-100nm). Our isolation technique produced exosome concentrations consistently to the magnitude of $1x10^{11}$ particles per ml plasma. Of interest, our NTA analysis results showed the presence of some larger particles in our sample (100-300nm), though in much smaller numbers. These larger particles may be microvesicles, plasma proteins, or potentially other subcellular organelles. A potential limitation of NTA is that it is unable to distinguish vesicles from other similarly sized particles, but when our NTA data is taken in combination with EM and western blotting results, it strongly indicates the presence of high quantities of exosomes. To provide further confidence in the use of the NTA machine as a quantification tool we tested the variability of the NTA analysis machine on our samples. Inter-sample variation on taking dilutions for nanosight analysis was well within acceptable limits with a variability

coefficient of 2.4%. Having gained confidence in the NTA as a quantification tool for our human plasma exosomes we were able to demonstrate that our isolation technique for human plasma exosomes was also consistent and the variability (variability coefficient 8.6%) in exosome numbers produced from our isolation technique was within acceptable limits. This provides confidence that we had established a reliable and reproducible method of exosome isolation from human plasma. Lastly while working with limited resources of human samples as well as the time consuming protocol for isolation, it was important to establish the optimal storage conditions for exosomes that would allow for future experiments. Using the NTA machine we were able to show that the optimal storage conditions for exosome samples were at -80°C, which was consistent with other literature findings [252,253].

As further evidence to demonstrate the presence of exosomal markers on the surface of isolated exosomes we utilized flow cytometric analysis. As evident from our western blotting experiments, protein and biomarker detection in exosomes themselves can be problematic due to the low protein yield and the probable lack of sensitivity of primary antibodies. Although flow cytometry is incapable of directly detecting particles of less than ~300 um including exosomes, we were able to make use of an alternative approach by adsorbing exosomes onto small, latex, microsphere beads[244]. In principle this technique should only detect proteins that lie on the surface of exosomes, so can also provide information as to the location of proteins, however we hoped that this technique would allow for easier biomarker detection and therefore provide semi-quantitative tool in exosomal analysis. We successfully demonstrated by flow cytometric analysis the detection of classical exosomal proteins CD63, CD9, HSP70 and CD81. Even the tetraspanin proteins, CD9 and CD81 that were not detectable via western blotting, using the same antibodies, were detected by our flow cytometry analysis. These results therefore support our hypothesis that flow cytometric analysis may be a more sensitive method for biomarker analysis of human plasma exosomes - certainly for those biomarkers or proteins that reside on the surface of the exosome.

Lastly we aimed to establish an isolation method to detect miRNA from our human plasma exosomes. The extent to which plasma miRNA is contained within exosomes is still controversial. Some studies suggest that exosomes contain the majority of plasma miRNA[197], while others find plasma miRNAs are mainly in argonaute complexes[198]. However, numerous studies have demonstrated the ability of exosomes to transfer miRNA to other cells [195,199] and miRNA have been implicated in both ischaemia reperfusion injury and heart failure[234]. The ability to regulate specific miRNA and their function could present a novel cardio-protective strategy. A reliable isolation protocol

for miRNA from human plasma exosomes would be required for future exploration of their potential role in cardio-protection in RIPC. Though in small quantities we were able to successfully isolate miRNA from our human plasma exosome samples. We observed signals from human plasma exosome samples for miR-16, snoU6 and GAPDH. UBC was expressed at close enough levels to be used as normalization control for GAPDH. SnoU6 is a better normalization control for our human exosome samples while RNU6B was not expressed at all in our samples. These results provide a protocol for further miRNA experiments on human plasma exosomes. Experiments comparing the miRNA content of RIPC and control exosomes can now be carried out. However we must exercise some caution. Extracting miRNA from exosomes relies critically on ensuring as pure a sample of exosomes as possible. From our previous western blot and nanosight experiments we are aware that there are still some serum contaminants in our sample. We have to be sure that miRNA observed in any further array testing is indeed coming from our exosomes and not contaminant protein. It is likely that improved isolation protocols will be identified in future, which may be a more appropriate exosome isolation protocol to then try and isolate microRNA from[252,253].

The mechanism of cardio-protection conferred through remote ischaemic preconditioning is not fully understood. Humoral factors are proposed to play a key role, yet the identification of the precise humoral factors have been challenging. Indeed it may be several humoral factors working in conjunction that lead to the cardio-protection seen through RIPC. Exosomes have the potential to ferry proteins or groups of protein to a target organ or cell and thus confer cardio-protection. With an isolation protocol established we aimed to explore further the relationship between RIPC and circulating plasma exosomes in humans. In the following experiments we examined the effect the remote ischaemic preconditioning had on human plasma exosomes, both in terms of their quantity as well as content. Our first set of experiments looked at the effect of RIPC on the concentration of human plasma exosomes using NTA analysis. Our results show interestingly that remote ischaemic preconditioning consisting of three cycles of forearm cuff inflation and deflation consistently lead to a rise in plasma exosome concentration in all participants. This trend was also seen in the parallel rat exosome experiments carried out within our research group. RIPC human plasma exosomes were also shown to contain greater concentrations of exosomal proteins HSP70 and CD63. This compliments our nanosight data and supports the conclusion that RIPC indeed leads to a rise in human plasma concentrations. Aware that platelet activation can also stimulate the release of exosomes as well as microvesicles, the exosome isolation method was developed to minimize platelet activation as much as possible. Additionally preconditioning has been shown to decrease platelet activation,

in particular limb RIPC has been shown to prevent systemic platelet activation in humans[164,254,255]. This gives us confidence that the rise in plasma exosomes post RIPC is unlikely to be attributed to platelet activation. The rise in exosome concentration post RIPC stimulus may indicate a role for exosomes in the cardio protection that is conferred through RIPC. If human plasma exosomes are indeed cardio-protective, does RIPC confer its cardio-protection through increasing circulating plasma exosome concentrations, which then exert their influence on the myocardium? Further work is needed to determine whether the increase in exosome concentration is causal in the induction of RIPC, or whether it is an unrelated phenomenon. Answering this question at present is challenging. An interesting experiment could involve an in-vivo rat model of RIPC where one administers a drug or compound that blocks exosome release just prior to the RIPC protocol. Loss of cardio-protection that one normally associates with RIPC would suggest indeed the rise in exosome concentration post RIPC is integral in the mechanism behind RIPC cardio-protection. Unfortunately at present no such compound exists that selectively blocks exosome release, though there is some interesting progress within this area with Rab proteins being implicated in exosome release[185]. Further experiments revealed that peak rise in exosome concentrations did not occur immediately after the last cuff deflation (reperfusion) in the 3 cycles of RIPC protocol, but after a further 5 min of reperfusion. This suggests that the exosomes are not simply washed out of the limb post-ischaemia, but are released during the reperfusion period. The convention of using 3 cycles of 5 minutes of cuff inflation and deflation in clinical and laboratory studies of RIPC both is not particularly based on scientific evidence, but was chosen for historical reasons based on parallels with direct ischaemic preconditioning. There have not been any studies that have looked in detail whether fewer cycles confer a similar protection. Assuming exosomes are causal in RIPC, our results may anecdotally suggest that 3 cycles of 5 min f cuff inflation and deflation are sufficient for induction of RIPC. However, no comparison was made with higher numbers of cycles of RIPC. Though beyond the scope of this project further experiments could be conducted to ascertain the precise point of rise in exosome concentration following RIPC. Could indeed one cycle 5 minutes of cuff inflation and deflation be enough to produce a similar response? It would also be interesting to determine the response in human plasma exosome concentration to further cycles of RIPC.

An alternative hypothesis for the role of plasma exosomes in the cardio-protective mechanism of RIPC involves the release of human plasma exosomes post RIPC stimulus with specific cardio-protective cargo. RIPC has also been shown to protect via the pro-survival protein kinases of the RISK pathway. Traditionally these pro-survival

kinases are activated through G protein coupled receptors (GPCR). We looked to investigate whether particularly RIPC plasma exosomes these active pro-survival protein kinases of the RISK pathway and therefore confer cardio protection directly when released in to the cell. Through western blotting and FACS analysis we probed both RIPC and control human plasma exosomes for PI3-Kinase, AKT, MEK1/2, and ERK1/2. Despite multiple attempts, we could not demonstrate the presence of pro-survival kinases directly in human plasma control or RIPC exosomes. This may simply be a limitation of working with samples that contain such a small amount of protein. However if the result is true, and indeed pro-survival kinases are not directly located in human plasma exosomes (especially RIPC exosomes), there may be other signalling proteins or growth factors within human plasma exosomes that may work at a higher level in the RISK pathway, and directly on the GPCR. Indeed HSP70 itself has been shown to have cardio-protective properties[249–251], however this protein is seen in both RIPC and control exosomes. This could possibly explain a mechanism for cardio-protection through plasma exosomes in general, however would not explain an exosomal mechanism for the cardio-protection conferred through RIPC as HSP70 is seen in both RIPC and control exosomes. Therefore one may conclude that RIPC may not produce human plasma exosomes with specific cardio-protective cargo but simply increase the concentration of human plasma exosomes and with this increase in concentration results in cardio-protection.

Our primary aim for this project centred on the hypothesis that human plasma exosomes play a mechanistic role in the cardio-protection conferred through RIPC. In particular we hypothesised that RIPC involves the release of human plasma exosomes with specific cardio-protective cargo. To test this hypothesis both RIPC and control exosomes were added to an in-vitro model of IR injury using rat cardio-myocytes. If our hypothesis were true one would expect RIPC plasma exosomes to confer cardio-protection while control plasma exosomes will not. The results of our experiments show that both the addition of 5µl of RIPC exosomes or control exosomes at reperfusion conferred significant cardio-protection and reduced cardiomyocyte cell death to 17 ± 3% and 17 ± 4% respectively. A dose response experiment with the addition of serial dilutions of RIPC and control exosomes at reperfusion demonstrated that the addition of 0.05µl and above of both isolated control and RIPC plasma exosomes confer cardio-protection against simulated our ischaemia reperfusion. Our results therefore demonstrate that plasma exosomes are cardio-protective against IR injury irrespective if they are control or RIPC plasma exosomes. The protection conferred by plasma exosomes are comparable to Insulin, a known cardio-protective agent, used here as our positive control. These results suggest that plasma exosomes in general are

cardio-protective and may not have a mechanistic role in the cardio-protection conferred through RIPC. There is some divergence in the degree of cardio-protection with the addition of 0.005µl of control and RIPC plasma exosomes. At this concentration RIPC exosomes still provide some degree of cardio-protection yet the control plasma exosomes do not. This however may simply be due to the lower concentration of exosomes in the control sample. We know from our previous experiment that RIPC exosome samples have a higher concentration of exosomes. Tentatively this may support our previous hypothesis that RIPC may lead to a rise in serum plasma exosome concentrations above and beyond a threshold that results in cardio-protection. Although not quite proving our initial hypothesis we have been able to show that human circulating plasma exosomes are cardio-protective. Its role in RIPC is yet to be established. A potential further experiment to add weight to our discovery would be to additionally add the exosome free plasma supernatant in to our in-vitro model of ischaemia reperfusion injury. If plasma deplete of exosomes failed to cardio-protect this would provide further strong evidence for their role in cardio-protection. One could confirm an exosome free supernatant through nanosight appearance as well as the lack of a positive signal of CD63 on a western blot. Adding human plasma to rat cardiomyocytes has obvious limitations. A further interesting experiment would also be to add human plasma exosomes in to a human atrial trabeculae model of ischaemia reperfusion and testing if similar cardio-protection is seen.

Recently Ferdinandy and colleagues published a paper exploring a similar hypothesis to this project. Using the effluent model of RIPC, perfusate from rats undergoing 3 × 5-5 min global ischemia and reperfusion (IPC) were depleted of exosomes by differential ultracentrifugation and was then given to another set of recipient isolated hearts. Interestingly there results also describe a marked increase in exosome release following an IPC stimulus. Additionally they showed that exosome deplete perfusate failed to exert cardio-protection in recipient hearts when compared to normal IPC perfusate. The group concludes exosomes released from the heart after IPC are necessary for cardio-protection by RIPC suggesting cardio-specific cargo in exosomes[256226]. This is in contrast to our results where RIPC exosomes exert similar cardio-protection to control exosomes. On further reflection an explanation for this disparity may be that RIPC is a complex cascade of interactions between blood cells and neuronal signals that the effluent model of RIPC does not truly capture. Additionally the effluent model perfusate will also be considerably more dilute than blood thus the concentration of exosomes would likely be much less. It may be that IPC increases the concentration of exosomes in the perfusate above a threshold needed to provide cardio-protection. Interestingly taken together our findings as well as

Ferdinandy and colleagues, the cardio-protective properties of plasma exosomes are in contrast to those of microvesicles that appear to have detrimental effects in RIPC[257].

The mechanism by which circulating plasma exosomes exert cardio-protection is yet to be identified and therefore must be the focus of future work within our group. Cardio-protection is seen rapidly after exosome perfusion suggesting an immediate mechanism. Upon analysis of both control and RIPC exosomes we could not demonstrate the presence of pro-survival kinases directly in human plasma control or RIPC exosomes. There may be other signalling proteins or growth factors within or on the surface of human plasma exosomes that interact directly with the plasma membrane and GPCR in the myocardium which then in turn activate further downstream traditional cardio-protective signalling pathways such as the RISK pathway described previously. There is already an established relationship between the RISK pathway and exosomes. Mesenchymal stem cell (MSC) exosomes have been shown to confer their cardio-protection via the RISK pathway. MSC exosomes have been shown increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway[258]. Additionally cisplatin-induced oxidative stress kidney injury was attenuated by exosomes from human umbilical cord mesenchymal stem cells protecting via ERK1/2[256]. A simple experiment to ascertain if the RISK pathway is involved in the mechanism of protection from plasma exosomes would be to western blot the cardiomyocytes, both with and without the addition of exosomes, for AKT and ERK. Increased signals of either would suggest that cardio-protection from exosomes follows traditional pro-survival pathways.

Identifying the potential signalling protein or growth factor that confers cardio-protection in plasma exosomes is the next challenge. HSP70 is a potential candidate. HSP70 is found in all prokaryotes and eukaryotes. Initially thought to be chaperone proteins, which facilitate the folding naïve, and refolding of denatured proteins aiding protein trafficking. Previously thought to largely intracellular, HSP70 is secreted and released outside cells by exosomes after stressful stimuli and the cardio-protective properties of HSP70 are also well established[249–251]. Could the release of HSP70 from exosomes lead to the cardio-protection seen in our invitro survival experiments? To test this hypothesis one could add a HSP70 inhibitor in to the invitro survival experiments along with the circulating plasma exosomes. If cardio-protection were lost this would suggest a role for HSP70. Other possibilities include proteins such as Il-10, CGRP and Stromal Derived Factor-1α (SDF-1α). Work from within our group has recently shown that RIPC results in an increase in circulating levels of SDF-1α. Additionally the blocking of SDF-1α using CXCR4 results in the attenuation of cardio-protection conferred by RIPC[86].

Could plasma exosomes contain SDF-1α? Exosomes may indeed ferry a number of pro-survival proteins and growth factors and protect via multiple mechanisms. MicroRNA are another potential cardio-protective candidate located within plasma exosomes. Though unlikely to be involved in immediate setting of ischaemia preconditioning, there maybe a role in the mechanism behind delayed or “second – window” of preconditioning.

Conclusion

In conclusion we have developed a viable and reproducible protocol for the isolation and analysis of human plasma exosomes. We have also shown that RIPC leads to a rise in circulating plasma exosomes and importantly plasma exosomes appear to be cardio-protective against ischaemia reperfusion injury. Exosomes therefore represent a new frontier in cardio-protection and further investigations are needed.

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Appendix